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# METHODS OF DIFFERENTIATING AND PROTECTING CELLS BY MODULATING THE P38/MEF2 PATHWAY

by

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# METHODS OF DIFFERENTIATING AND PROTECTING CELLS BY MODULATING THE P38/MEF2 PATHWAY

This application is based on, and claims the benefit of, U.S. Provisional Application No. 60/209,539, filed June 5, 2000, and which is incorporated herein by reference.

This application was made with government support under P01 HD29587 awarded by the National Institute of Health. The government has certain rights in the invention.

## BACKGROUND OF THE INVENTION

### FIELD OF THE INVENTION

The invention relates to neuronal cell transplantation for neurodegenerative conditions of the central nervous system including hypoxia-ischemia (stroke), Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease and other forms of dementia and, more specifically, to methods of producing populations of neurons by manipulating the myocyte enhancer factor 2 (MEF2) transcription pathway.

## BACKGROUND INFORMATION

For a variety of serious neurodegenerative diseases, there exist no effective therapies or cures. For example, Parkinson's disease is a progressive and ultimately fatal neurodegenerative disorder characterized by loss of the pigmented dopaminergic neurons of the *substantia nigra*. The symptoms of Parkinson's disease can often be managed initially by administration of L-DOPA, the immediate precursor of dopamine. However, reduced efficacy of L-DOPA treatment typically occurs over time. Programmed cell death (apoptosis) has been implicated in this neurodegenerative disorder.

In Alzheimer's disease, the most common neurodegenerative disease and most frequent cause of dementia, progressive failure of memory and degeneration of temporal and parietal association cortex result in speech impairment and loss of coordination, and, in some cases, emotionally disturbance. Alzheimer's disease generally progresses over many years, with patients gradually becoming immobile, emaciated and susceptible to pneumonia.

The brain constitutes a privileged transplantation site and, under the appropriate conditions, neuronal tissues can survive transplantation into the damaged brain, integrate with the host and alleviate functional impairments associated with neurological disease. Neuronal cell transplantation has been sought for a variety of serious neurodegenerative diseases for which no effective therapeutic course exists, including Parkinson's disease and Alzheimer's

disease as well as Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, epilepsy and pain.

Present techniques for neuronal transplantation have chiefly relied on embryonic or fetal tissues since central nervous system (CNS) neurons only survive transplantation if taken from embryonic or neonatal donors. Neuronal transplantation has been hampered by extremely limited supplies of human embryonic or fetal tissue. In order to develop alternative supplies of donor neurons, scientists have attempted the large scale expansion of stem cells and precursor cells. When treated with high doses of epidermal growth factor, stem and precursor cells from the brain can be selectively expanded *in vitro* and grown exponentially through multiple passages. These expanded cells, which can be produced from human tissues, yield both neuronal and glial cell types when allowed to differentiate *in vitro* and survive transplantation back into animal central nervous system (CNS; Svendsen et al., Exp. Neurol. 140:1-13 (1996)).

Unfortunately, the expansion of stem and precursor cell populations currently does not produce a cell population useful for therapeutic transplantation, since a relatively small number of neurons is produced, and even a smaller number survive and express the neuronal phenotype when grafted into the central nervous system. Thus, there is a need for a method of efficiently producing large numbers of neuronal cells or their precursors which are capable of surviving when transplanted into the central nervous system *in vivo*.

The present invention satisfies this need and provides related advantages as well.

## SUMMARY OF THE INVENTION

The present invention provides a method of differentiating progenitor cells by contacting the progenitor cells with a differentiating agent; and introducing into the progenitor cells a nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof, thereby differentiating the progenitor cells to produce a cell population containing protected neuronal cells. In one embodiment, the produced population containing protected neuronal cells contains at least 50% neuronal cells.

A method of the invention can be practiced, for example, with a nucleic acid molecule encoding human MEF2C, or an active fragment thereof. In one embodiment, the MEF2 polypeptide is constitutively active. In another embodiment, the constitutively active MEF2 polypeptide is a MEF2/VP16 fusion protein. In a further embodiment, the constitutively active MEF2 polypeptide contains one or more serine/threonine to aspartic acid/glutamic acid substitutions in the MEF2 transactivation domain.

A method of the invention for differentiating progenitor cells to produce a cell population containing protected neuronal cells can further include the step of inhibiting caspase activity in the progenitor cells.

Progenitor cells useful in the methods of the invention can be, for example, human stem cells. In one embodiment, the progenitor cells are embryonic stem cells, for example, human embryonic stem cells. In another embodiment, the progenitor cells are hematopoietic progenitor cells, for example, human hematopoietic progenitor cells.

In one embodiment, a method of the invention further includes the step of selecting CD133-positive (AC133-positive) human progenitor cells. In another embodiment, a method of the invention includes the step of selecting CD133-positive/CD34-positive human progenitor cells. In a further embodiment, a method of the invention further includes the step of selecting CD133-positive/ CD34-negative human progenitor cells. In yet further embodiments, CD133-positive/CD34-negative/ CD45-negative, or CD34-negative/CD38-negative/ Lin-negative human progenitor cells or CD34-positive/CD38-negative/ Lin-negative/ Thy-1-negative human progenitor cells are selected.

For use in a method of the invention, the differentiating agent can be, for example, retinoic acid. Other differentiating agents useful in a method of the invention for producing a cell population containing protecting neurotrophic factor 3, epidermal growth factor, insulin-like growth factor 1 and a platelet-derived growth factor.

In a further embodiment, a method of the invention further includes the step of transplanting cells containing a nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof into a patient to produce a cell population containing protected neuronal cells in the patient.

The invention further provides an isolated stem cell that contains an exogenous nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof. The isolated stem cell can include, for example, a nucleic acid molecule encoding a MEF2 polypeptide, or active fragment thereof, operatively linked to a heterologous regulatory element. The encoded MEF2 polypeptide can be, for example, a human MEF2 polypeptide. If desired, the encoded MEF2 polypeptide can be a MEF2C polypeptide. In a further embodiment, the MEF2 polypeptide is constitutively active. Such a constitutively active MEF2 polypeptide can be, for example, a constitutively active MEF2C polypeptide. In one embodiment, the constitutively active MEF2 polypeptide is a MEF2/VP16 fusion protein. In another embodiment, the constitutively active MEF2 polypeptide contains one or more serine/threonine to aspartic acid/glutamic acid substitutions in the MEF2 transactivation domain.

An isolated stem cell of the invention can be, for example, a human stem cell. In one embodiment, the stem cell is an embryonic stem cell, for example, a human embryonic stem cell. A human stem cell of the invention can contain, for example, an exogenous nucleic acid molecule encoding human MEF2C. A human stem cell of the

invention also can include a constitutively active MEF2 polypeptide.

The invention further provides an isolated hematopoietic stem cell that contains an exogenous nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof. Such an isolated hematopoietic stem cell can include, for example, a nucleic acid molecule encoding a MEF2 polypeptide, or active fragment thereof, operatively linked to a heterologous regulatory element. In one embodiment, an isolated hematopoietic stem cell of the invention is a human hematopoietic stem cell.

The present invention also provides a method of identifying a protective or differentiation gene, which can be, for example, a neuroprotective gene or a gene that contributes to neuronal or muscle cell differentiation. A method of the invention includes the steps of isolating a first cell population; isolating a second cell population, wherein the second cell population has an altered level or activity of a MEF2 polypeptide as compared to the first cell population; and assaying for differential gene expression in the first cell population as compared to the second cell population, whereby a gene differentially expressed in the second cell population as compared to the first cell population is identified as a protective or differentiation gene. In one embodiment, the first cell population is a progenitor cell population, the second cell population is a neuronal cell population, and the differentially expressed gene is a neuronal differentiation gene. In a further embodiment, the first

cell population is a progenitor cell population, the second cell population is a muscle cell population, and the differentially expressed gene is a muscle differentiation gene. In yet another embodiment, both cell populations are neuronal cell populations, the second cell population has been subject to a neuronal stress as compared to the first cell population, and the differentially expressed gene is a neuroprotective gene.

Further provided by the invention is a method of identifying a protective gene *in vitro*. The method is practiced by inducing the p38/MEF2 pathway in a cell *in vitro* to produce a protected cell; stressing the cell; and assaying for differential gene expression in the protected cell as compared to gene expression in a control cell, whereby a gene differentially expressed in the protected cell as compared to the control cell is identified as a protective gene. In such a method of the invention, the p38/MEF2 pathway can be induced, for example, by introducing into the cell a nucleic acid molecule encoding a MEF2 polypeptide. The MEF2 polypeptide can be, for example, a human MEF2 polypeptide and further can be, if desired, a constitutively active MEF2 polypeptide. In one embodiment, a neuroprotective gene is identified by inducing the p38/MEF2 pathway in a neuron. In another embodiment, a muscle protective gene is identified by inducing the p38/MEF2 pathway in a muscle cell. In a method of the invention, the differential gene expression that identifies the protective gene can be increased or decreased gene expression.

The invention additionally provides a method of identifying a differentiation gene *in vitro* by inducing the p38/MEF2 pathway in a progenitor cell *in vitro* to produce a differentiated cell; and assaying for differential gene expression in the differentiated cell as compared to gene expression in a control cell, whereby a gene differentially expressed in the differentiated cell as compared to the control cell is identified as a differentiation gene. In a method of the invention, the p38/MEF2 pathway can be induced, for example, by introducing into the progenitor cell a nucleic acid molecule encoding a MEF2 polypeptide. The MEF2 polypeptide can be, for example, a human MEF2 polypeptide or a constitutively active MEF2 polypeptide. In one embodiment, the differentiated cell is a neuronal cell, and, in a further embodiment, the differentiated cell is a muscle cell. The differential gene expression which serves to identify the differentiation gene can be increased or decreased gene expression.



## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1*A* shows schematic diagrams of MEF2 factors. Structures of the four vertebrate mef2 gene products are shown. Alternative exons within the C-terminal activation domains are indicated, along with the number of amino acids in the longer form of each protein. Figure 1*B* shows conserved regions in the C terminus of MEF2 contain potential phosphorylation sites. MEF2A motifs 1 to 4 are shown as SEQ ID NOS: 13 to 16, respectively. MEF2C motifs 1 to 4 are shown as SEQ ID NOS: 17 to 20, respectively. MEF2D motifs 1 to 3 are shown as SEQ ID NOS: 21 to 23, respectively. Sites that

are phosphorylated by p38 and ERK5 are marked with asterisks. Not all potential phosphorylation sites are shown. Some of the conserved stretches overlap with transactivation domains that have been mapped by deletion analysis. PKC, protein kinase C site: MAPK, mitogen-activated protein kinase site; CKII casein kinase II site. CaMKIV, calcium-calmodulin kinase IV site. MEF2A motifs 1 to 4 are shown as SEQ ID NOS: 13 to 16, respectively. MEF2C motifs 1 to 4 are shown as SEQ ID NOS: 17 to 20, respectively. MEF2D motifs 1 to 3 are shown as SEQ ID NOS: 21 to 23, respectively.

Figure 2*A* shows the nucleotide sequence (SEQ ID NO: 1) of human MEF2A (GenBank accession NM 005587). Figure 2*B* shows the amino acid sequence (SEQ ID NO: 2) of human MEF2A.

Figure 3*A* shows the nucleotide sequence (SEQ ID NO: 3) of human MEF2B (GenBank accession NM 005919). Figure 3*B* shows the amino acid sequence (SEQ ID NO: 4) of human MEF2B.

Figure 4*A* shows the nucleotide sequence (SEQ ID NO: 5) of human MEF2C (GenBank accession L08895). Figure 4*B* shows the amino acid sequence (SEQ ID NO: 6) of human MEF2C. The MADS domain is bolded while the MEF2 domain is underlined.

Figure 5*A* shows the nucleotide sequence (SEQ ID NO: 7) of human MEF2D (GenBank accession NM 005920). Figure 5*B* shows the amino acid sequence (SEQ ID NO: 8) of human MEF2D.

Figure 6 shows MEF2 binding activity, protein expression and transfection during neuronal differentiation of P19 stem cells. (*A*) Gel shift assays show that MEF2 binding activity increased during neuronal differentiation of P19 cells. A $^{32}$P-labeled MEF2 site oligonucleotide was incubated with nuclear extracts from undifferentiated P19 cells (lanes 1 and 3), or from P19 cells treated with retinoic acid for 2 d (lanes 2 and 4). Cold competition by unlabeled MEF2 site oligonucleotides (lanes 3 and 4). (*B*) Antibody to MEF2A (lane 6), MEF2C (lane 7), or MEF2D (lane 8) was added to the binding mixture for supershift assays. Anti-MEF2C yielded two supershifted bands, representing one or more DNA complexes containing MEF2C, while anti-MEF2D produced a single supershifted complex (arrows; Leifer et al., Proc. Natl. Acad. Sci. USA 90:1546-1550 (1993)). (*C*) Immunoblots revealed that protein expression of MEF2C and MEF2D was induced during neuronal differentiation of P19 cells. Whole cell lysates from undifferentiated P19 cells or P19 cells treated with retinoic acid for 2 days were used for these immunoblots (n.s., non-specific bands). (*D-G*) Overexpression of MEF2C induced a mixed neurogenic/myogenic phenotype. Undifferentiated P19 cells did not display immunoreactivity for MEF2C or neurofilament (*D*, phase contrast image; *E*, immunocytochemistry). Undifferentiated P19 cells were transfected with an expression vector for MEF2C. Similar findings were observed in 16 experiments in which over 200 cells were scored.

Figure 7 shows inhibition of MEF2 function decreases the number of neuronal (MAP2-positive) P19 cells after retinoic acid treatment. (*A*) Undifferentiated

P19 cells were stably transfected with empty vector (clones 2-1, 2-3 and 2-5 in lanes 1-3, respectively) or MEF2 dominant negative (clones 2-7, 2-8 and 2-9 in lanes 4-6, respectively). Expression of the MEF2 dominant negative was demonstrated in a gel shift assay using a radiolabeled MEF2 site oligonucleotide and nuclear extracts of each clone. DN: binding complex of the MEF2 site and dominant negative MEF2 protein. (*B* and *C*) Cultures from control (*B*, clone 2-1) and MEF2 dominant negative (*C*, clone 2-7) transformants were treated with retinoic acid for 7 d to induce neurogenesis, and neuronal differentiation was then evaluated by immunocytochemistry with anti-MAP2. (*D*) Control cultures (clones 2-1, 2-3, 2-5) and MEF2 dominant negative cultures (clones 2-7, 2-8, 2-9) were treated with retinoic acid and scored for the number of MAP2-positive cells ($n$ = 6 experiments; *, $P < 0.0001$ by ANOVA and post-hoc comparison).

Figure 8 shows that inhibition of MEF2 function decreases the number of multipotent and unipotent precursor cells. Control cultures (clone 2-1) and MEF2 dominant negative cultures (clone 2-7) were treated with retinoic acid for 3.0 d or 3.5 days. (*A* and *C*) Cells incubated with anti-nestin to label multipotent precursor cells (*A*) or anti-Hu to label unipotent precursor cells (*C*). Labeled cells were visualized with peroxidase. (*B* and *D*) The number of nestin-positive cells (*B*) and Hu-positive cells (*D*) in 40 randomly selected fields was scored in a blinded fashion. Values are mean ± SD from at least three independent experiments (*, $P < 0.02$ by Student's $t$-test).

Figure 9 shows the effects of inhibition of MEF2 function during neuronal differentiation of P19 cells. Control cultures (clone 2-1), MEF2 dominant negative cultures (clone 2-7, labeled DN) and mutated MEF2 dominant negative cultures (clone 2-16, labeled DNmt) were treated with retinoic acid for 3 days. (*A*) Representative apoptotic cells with condensed nuclei from a MEF2 dominant negative clone treated with retinoic acid and stained with Hoechst dye to detect apoptotic morphology (white arrows). (*B*) Percentage of apoptotic cells in control or MEF2 dominant negative cultures before and after retinoic acid treatment. (*C*) Similar percentage of apoptotic cells in control or mutated MEF2 dominant negative cultures after 3 days of retinoic acid. (*D*) Apoptosis in control, dominant negative or mutated dominant negative cultures treated with retinoic acid for 3 days scored by the TUNEL technique. (*E* and *F*) Lack of effect of MEF2 dominant negative on multipotent precursor cell proliferation. Control cultures and MEF2 dominant negative cultures were treated with retinoic acid for 3 days. BrdU was then added to visualize proliferating cells. (*E*) Dividing multipotent precursor cells detected by double staining with anti-BrdU antibody and anti-nestin antibody in retinoic acid-treated control cells. (*F*) Comparison of BrdU incorporation into multipotent (nestin-positive) precursor cells in control and MEF2 dominant negative cultures. Values are mean ± SD from at least three independent experiments (*, $P < 0.05$ by Student's *t*-test; †, $P < 0.001$ by ANOVA and post-hoc comparison).

Figure 10 shows involvement of the p38α/MEF2 pathway in preventing apoptosis during neuronal

differentiation of P19 cells. (*A*) p38α was phosphorylated during induction of neuronal differentiation by retinoic acid. Anti-phospho p38 was used to detect activated/phosphorylated p38 family members on immunoblots during induction of neuronal differentiation. The same membrane was then stripped and re-blotted with a p38α-specific antibody that labeled the same band. (*B*) Dominant negative p38α (p38αDN) enhanced apoptosis during neuronal differentiation. Constitutively active MEF2C (MEF2C/VP16) significantly rescued the differentiating cells from apoptosis. Dominant negative p38β (p38b DN) had no effect on apoptosis compared to control (expression vector only). After treatment with retinoic acid for one day, cells were transfected with the indicated expression vector(s) along with a GFP expression vector to identify the transfected cells. The number of transfected apoptotic cells was determined in a blinded fashion by TUNEL assay on day 3 of retinoic acid treatment. Over 1200 GFP-positive cells were scored in each culture. Mean ± SD are shown from three experiments (*, $P < 0.001$; †, $P < 0.01$ by ANOVA and post-hoc comparison).

## DETAILED DESCRIPTION OF THE INVENTION

MEF2 proteins are members of the MADS family of transcription factors (Treisman, R., Nature 376:468-469 (1995)). Four members of the family (MEF2A, MEF2B, MEF2C and MEF2D) have been reported, including murine and human polypeptides (Pollock and Treisman, Genes & Dev. 5:2327-2341 (1991); Leifer et al., Proc. Natl. Acad. Sci. USA 90:1546-1550 (1993); Martin et al., Proc. Natl. Acad. Sci. USA 90:5282-5286 (1993); Martin et al., Mol. Cell.

Biol. 14:1647-1656 (1994); and Molkentin et al., Mol. Cell. Biol. 16:3814-3824 (1996)), and one MEF2 homolog, D-MEF2, has been identified in *Drosophila* (Lilly et al., Proc. Natl. Acad. Sci., USA 91:5662-5666 (1994)). Various MAP kinases (p38α, p38β, and BMK1/ERK5) phosphorylate and thereby activate MEF2 family members (Han et al., Nature 386:296-299 (1997); Kato et al., EMBO J. 16:7054-7066 (1997); Zhao et al., Mol. Cell. Biol. 19:21-30 (1999); and Yang et al., Mol. Cell. Biol. 19:4028-4038 (1999)). These MAP kinase pathways lead to MEF2 modulation of gene expression (Han et al., Nature 386:296-299 (1997); Kato et al., EMBO J. 16:7054-7066 (1997); and Zhao et al., Mol. Cell. Biol. 19:21-30 (1999)).

The MEF2 family of genes is highly expressed in cells of muscle lineage, and several studies support a role for MEF2 in myogenesis (Martin et al., Proc. Natl. Acad. Sci. USA 90:5282-5286 (1993); Martin et al., Mol. Cell. Biol. 14:1647-1656 (1994); Molkentin et al., Mol. Cell. Biol. 16:3814-3824 (1996); and McDermott et al., Mol. Cell. Biol. 13:2564-2577 (1993)). In *D-mef2* loss-of-function *Drosophila*, muscle cells have lost the ability to differentiate (Lilly et al., Science 267:688-693 (1995)), and MEF2C-null mice are embryonic lethal due to malformation of the heart (Lin et al., Science 276:1404-1407 (1997)). Furthermore, a dominant negative form of MEF2 also inhibits myotube formation in myoblastic cell lines (Ornatsky et al., J. Biol. Chem. 272:33271-33278 (1997)). During myogenesis, evidence indicates that MEF2 proteins physically interact with the basic helix-loop-helix (bHLH) myogenic transcription factors MyoD and myogenin to initiate muscle development

(Kaushal et al., Science 266:1236-1240 (1994) and Molkentin et al., Cell 83:1125-1136 (1995)).

MEF2 family members including MEF2C also are highly expressed in neurons in the central nervous system (CNS) (Lyons et al., J. Neurosci. 15:5727-5738 (1995); Leifer et al., Proc. Natl. Acad. Sci. USA 90:1546-1550 (1993)). The level of MEF2 expression increases in differentiating neurons in the developing brain (Leifer et al., Proc. Natl. Acad. Sci. USA 90:1546-1550 (1993); Lyons et al., J. Neurosci. 15:5727-5738 (1995); Leifer et al., Neuroscience 63:1067-1079 (1994); and Lin et al., Mol. Brain. Res. 42:307-316 (1996)). Several neuronal bHLH transcription factors have been identified during mammalian development (Lee et al., Mol. Cell. Biol. 17:2745-2755 (1997)). Ectopic overexpression of neuronal bHLH factors NeuroD(1)/BETA2, NeuroD2/KW8/NDRF, or NeuroD3/neurogenin1 in *Xenopus* causes neurogenic conversion of ectoderm (Lee et al., Mol. Cell. Biol. 17:2745-2755 (1997)). In addition, physical and functional interaction between a neuronal bHLH transcription factor (Mash-1) and MEF2 proteins has been reported (Skerjanc and Wilton, FEBS Letters 472:53-56 (2000); Mao and Nadal-Ginard, J. Biol. Chem. 271:14371-14375 (1996); and Black et al., J. Biol. Chem. 271:26659-26663 (1996)).

The present invention relates to the finding that the p38α/MEF2 pathway plays an important role in preventing apoptotic cell death during neuronal differentiation. Based on this finding, the present invention provides a method of differentiating progenitor cells to produce a population of neuronal cells which is

protected from apoptotic cell death. The method includes the steps of contacting the progenitor cells with a differentiating agent and inducing the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (p38 MAP kinase/MEF2) pathway in the progenitor cells, thereby differentiating the progenitor cells to produce a population containing protected neuronal cells. In one embodiment, the MAP kinase/MEF2 pathway is induced by introducing into the progenitor cells a nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof. A MEF2 polypeptide useful in a method of the invention can be, for example, a human MEF2 polypeptide or an active fragment thereof and, in one embodiment, is a MEF2C polypeptide or active fragment thereof, for example, a human MEF2C polypeptide or active fragment thereof.

In another embodiment, a method of the invention for differentiating progenitor cells is practiced by introducing into the progenitor cells a nucleic acid molecule encoding a constitutively active MEF2 polypeptide such as a constitutively active form of MEF2A, MEF2B, MEF2C or MEF2D. In a further embodiment, the constitutively active form of the MEF2 polypeptide is resistant to caspase cleavage. A constitutively active MEF2 polypeptide useful in the invention can be, for example, a chimera in which the native MEF2 activation domain is replaced with a heterologous activation domain, for example, a constitutively active MEF2A/VP16, MEF2A/GAL4, MEF2B/VP16, MEF2B/GAL4, MEF2C/VP16, MEF2C/GAL4, MEF2D/VP16 or MEF2D/GAL4 fusion protein. A constitutively active MEF2 polypeptide useful in the invention also can be, for example, a modified MEF2

polypeptide in which one or all of the p38 kinase phosphorylation sites in the MEF2 transactivation domain are substituted with an aspartic or glutamic acid residue. In a further embodiment, a method of the invention is practiced by introducing into the progenitor cells a MEF2 activator, whereby the p38/MEF2 pathway is induced. Such a MEF2 activator can be, for example, a nucleic acid molecule encoding p38α.

In another embodiment, a method of the invention for differentiating progenitor cells to produce a population containing protected neuronal cells is practiced by introducing into the progenitor cells a caspase inhibitor in addition to administering the agent that induces the p38/MEF2 pathway. It is understood that the caspase inhibitor can be administered together with, prior to or following administration of the agent that induces the p38/MEF2 pathway. In one embodiment, a method of the invention is practiced by introducing into the progenitor cells a caspase inhibitor in addition to a constitutively active MEF2 polypeptide.

In a further embodiment, the MAP kinase/MEF2 pathway is induced by introducing into the progenitor cells a MEF2 activator such as a nucleic acid molecule encoding p38α. A population containing protected neuronal cells produced by a method of the invention can be made up of, for example, at least 50% neuronal cells. If desired, cells containing a nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof can be transplanted into a patient, for example, into the brain, central nervous system or retina, to produce a

cell population containing protected neuronal cells in the patient.

The present invention also provides a method of reducing the severity of a neurologic condition in a subject by administering to the subject an agent that induces the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (MEF2) pathway. A method of the invention can be useful, for example, in reducing the severity of an acute neurologic condition such as cerebral ischemia; stroke; hypoxia; anoxia; poisoning by carbon monoxide, manganese or cyanide; hypoglycemia; mechanical trauma to the nervous system such as trauma to the head or spinal cord; or epileptic seizure. A method of the invention further can be useful, for example, for reducing the severity of a chronic neurodegenerative disease such as Huntington's disease; a disorder of photoreceptor degeneration such as retinitis pigmentosa; acquired immunodeficiency syndrome (AIDS) dementia complex; a neuropathic pain syndrome such as causalgia or a painful peripheral neuropathy; olivopontocerebellar atrophy; Parkinsonism; amyotrophic lateral sclerosis; a mitochondrial abnormality or other biochemical disorder such as MELAS syndrome, MERRF, Leber's disease, Wernicke's encephalopathy, Rett syndrome, homocysteinuria, hyperhomocysteinemia, hyperprolinemia, nonketotic hyperglycinemia, hydroxybutyric aminoaciduria, sulfite oxidase deficiency, combined systems disease, lead encephalopathy; Alzheimer's disease, hepatic encephalopathy, Tourette's syndrome, or drug addiction, tolerance or dependency. Thus, in one embodiment, a method of the invention reduces the severity of stroke; hypoglycemia; trauma; epilepsy; neuropathic pain;

peripheral neuropathy, for example, associated with diabetes mellitus; glaucoma; multiple sclerosis. In another embodiment, a method of the invention reduces the severity of Alzheimer's disease, Huntington's disease, acquired AIDS dementia complex, or amyotrophic lateral sclerosis. In a further embodiment, a method of the invention reduces the severity of depression, anxiety, or drug dependency, drug withdrawal or drug addiction.

A method of the invention for reducing the severity of a neurologic condition can be practiced, for example, by administering to a subject a nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof. A MEF2 polypeptide useful in a method of the invention can be, for example, a human MEF2 polypeptide or an active fragment thereof and, in one embodiment, is a MEF2C polypeptide or active fragment thereof, for example, a human MEF2C polypeptide or active fragment thereof.

In another embodiment, a method of the invention for reducing the severity of a neurologic condition is practiced by administering to a subject a nucleic acid molecule encoding a constitutively active MEF2 polypeptide such as a constitutively active form of MEF2A, MEF2B, MEF2C or MEF2D. In a further embodiment, the constitutively active form of the MEF2 polypeptide is resistant to caspase cleavage. A constitutively active MEF2 polypeptide useful in the invention can be, for example, a chimera in which the native MEF2 activation domain is replaced with a heterologous activation domain, for example, a constitutively active MEF2A/VP16, MEF2A/GAL4, MEF2B/VP16, MEF2B/GAL4, MEF2C/VP16,

MEF2C/GAL4, MEF2D/VP16 or MEF2D/GAL4 fusion protein. A constitutively active MEF2 polypeptide useful in the invention also can be, for example, a modified MEF2 polypeptide in which one or all of the p38 kinase phosphorylation sites in the MEF2 transactivation domain are substituted with an aspartic or glutamic acid residue. In a further embodiment, a method of the invention is practiced by administering to a subject a MEF2 activator, whereby the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (MEF2) pathway is induced. Such a MEF2 activator can be, for example, a nucleic acid molecule encoding p38α.

In another embodiment, a method of the invention for reducing the severity of a neurologic condition by administering a caspase inhibitor in addition to administering the agent that induces the p38/MEF2 pathway. It is understood that the caspase inhibitor can be administered together with, prior to or following administration of the agent that induces the p38/MEF2 pathway. In one embodiment, a method of the invention is practiced by administering a caspase inhibitor in addition to a constitutively active MEF2 polypeptide.

The invention further provides a method of protecting a neuron from cell death by inducing in the neuron the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (MEF2) pathway. Such a neuron can be, for example, an adult neuron. A method of the invention can be useful, for example, in protecting a neuron from apoptotic cell death due to an insult such as NMDA receptor-mediated toxicity, or oxidative or nitrosative

stress. In one embodiment, a method of the invention for protecting a neuron from cell death is practiced by introducing into the neuron a nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof. A method of the invention can be practiced, for example, by introducing into a neuron a nucleic acid molecule encoding a human MEF2 polypeptide or an active fragment thereof and, in one embodiment, is practiced by introducing into a neuron a MEF2C polypeptide or active fragment thereof, for example, a human MEF2C polypeptide or active fragment thereof.

In another embodiment, a method of the invention is practiced by introducing into a neuron a nucleic acid molecule encoding a constitutively active MEF2 polypeptide such as a constitutively active form of MEF2A, MEF2B, MEF2C or MEF2D. In a further embodiment, the constitutively active form of the MEF2 polypeptide is resistant to caspase cleavage. A constitutively active MEF2 polypeptide can be, for example, a chimera in which the native MEF2 activation domain is replaced with a heterologous activation domain, for example, a constitutively active MEF2A/VP16, MEF2A/GAL4, MEF2B/VP16, MEF2B/GAL4, MEF2C/VP16, MEF2C/GAL4, MEF2D/VP16 or MEF2D/GAL4 fusion proteins. A constitutively active MEF2 polypeptide also can be, for example, a modified MEF2 polypeptide in which one or all of the p38 kinase phosphorylation sites in the MEF2 transactivation domain are substituted with an aspartic or glutamic acid residue. In yet a further embodiment, a method of the invention is practiced by introducing into a neuron a MEF2 activator in order to induce the p38 mitogen-activated protein kinase/myocyte enhancer

factor 2 (MEF2) pathway in the neuron. Exemplary MEF2 activators useful in the invention include p38α-encoding nucleic acid molecules.

In a further embodiment, a method of the invention for protecting a neuron from cell death is practiced by inducing the p38/MEF2 pathway and further introducing a caspase inhibitor into the neuron. It is understood that the caspase inhibitor can be administered together with, prior to, or following induction of the p38/MEF2 pathway. In one embodiment, a neuron is protected from cell death by introducing into the neuron a constitutively active MEF2 polypeptide and a caspase inhibitor.

The invention also provides a method of protecting a muscle cell from cell death by inducing in the muscle cell the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (MEF2) pathway. Such a muscle cell can be, for example, an adult muscle cell. A method of the invention can be useful, for example, in protecting a heart muscle cell from injury in a subject susceptible to heart attack (myocardial infarction). In one embodiment, a method of the invention for protecting a muscle cell from cell death is practiced by introducing into the muscle cell a nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof. A method of the invention can be practiced, for example, by introducing into the muscle cell a nucleic acid molecule encoding a human MEF2 polypeptide or an active fragment thereof. In one embodiment, a method of the invention is practiced by introducing into the muscle cell a MEF2C

polypeptide or an active fragment thereof, for example, a human MEF2C polypeptide or an active fragment thereof.

In another embodiment, a method of the invention is practiced by introducing into a muscle cell a nucleic acid molecule encoding a constitutively active MEF2 polypeptide such as a constitutively active form of MEF2A, MEF2B, MEF2C or MEF2D. In one embodiment, the constitutively active form of the MEF2 polypeptide is resistant to caspase cleavage. In yet a further embodiment, a method of the invention for protecting a muscle cell from cell death is practiced by introducing into the muscle cell a constitutively active MEF2 polypeptide in which the native MEF2 activation domain is replaced with a heterologous activation domain, for example, a constitutively active MEF2A/VP16, MEF2A/GAL4, MEF2B/VP16, MEF2B/GAL4, MEF2C/VP16, MEF2C/GAL4, MEF2D/VP16 or MEF2D/GAL4 fusion proteins. A constitutively active MEF2 polypeptide useful in protecting a muscle cell from cell death also can be, for example, a modified MEF2 polypeptide in which one or all of the p38 kinase phosphorylation sites in the MEF2 transactivation domain are substituted with an aspartic or glutamic acid residue. In yet another embodiment, a method of the invention is practiced by introducing into a muscle cell a MEF2 activator such as a p38α-encoding nucleic acid molecule in order to induce the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (MEF2) pathway in the neuron.

A method of the invention for protecting a muscle cell from cell death also can be practiced by inducing the p38/MEF2 pathway and further introducing a

caspase inhibitor into the muscle cell. It is understood that the caspase inhibitor can be administered together with, prior to, or following induction of the p38/MEF2 pathway. In one embodiment, a muscle cell is protected from cell death by introducing into the muscle cell a constitutively active MEF2 polypeptide and a caspase inhibitor.

The present invention further provides a method of generating muscle cells from progenitor cells by inducing in the progenitor cells the p38 mitogen-activated protein kinase/myocyte pathway and transplanting the cells into a muscle cell environment, thereby differentiating the progenitor cells to produce a population containing muscle cells. In one embodiment, the progenitor cells are differentiated to produce a cell population containing protected muscle cells. A method of the invention for generating muscle cells can be useful, for example, for generating cardiac muscle following myocardial infarction, congestive heart failure, cardiomyopathy or other injury to heart tissue. Where cardiac muscle is to be generated, the cells can be transplanted into the heart wall, which provides the proper tissue environment for muscle cell differentiation. In one embodiment, a method of the invention for protecting a muscle cell from cell death is practiced by introducing into the muscle cell a nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof.

A method of the invention for generating muscle cells can be practiced, for example, by introducing into a progenitor cell a nucleic acid molecule encoding a MEF2

polypeptide or an active fragment thereof. Such a MEF2 polypeptide can be, for example, a human MEF2 polypeptide. In one embodiment, a method of the invention for generating muscle cells is practiced by introducing into a progenitor cell a MEF2C polypeptide or an active fragment thereof, for example, a human MEF2C polypeptide or an active fragment thereof.

In another embodiment, a method of the invention is practiced by introducing into a progenitor cell a nucleic acid molecule encoding a constitutively active MEF2 polypeptide, which can be, for example, a constitutively active form of MEF2A, MEF2B, MEF2C or MEF2D. In a further embodiment, the constitutively active form of the MEF2 polypeptide is resistant to caspase cleavage. In yet a further embodiment, a method of the invention for generating muscle cells is practiced by introducing into a progenitor cell a constitutively active MEF2 polypeptide in which the native MEF2 activation domain is replaced with a heterologous activation domain. Exemplary constitutively active MEF2 polypeptides include, for example, MEF2A/VP16, MEF2A/GAL4, MEF2B/VP16, MEF2B/GAL4, MEF2C/VP16, MEF2C/GAL4, MEF2D/VP16 and MEF2D/GAL4 fusion proteins and modified MEF2 polypeptides in which one or all of the p38 kinase phosphorylation sites in the MEF2 transactivation domain are substituted with an aspartic or glutamic acid residue. In yet another embodiment, a method of the invention for generating muscle cells is practiced by introducing into a progenitor cell a MEF2 activator such as a p38α-encoding nucleic acid molecule in order to induce the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (MEF2) pathway in the neuron.

In a further embodiment, muscle cells are generated by inducing the p38/MEF2 pathway and further introducing a caspase inhibitor into the progenitor cells. It is understood that the caspase inhibitor can be administered together with, prior to, or following induction of the p38/MEF2 pathway. In one embodiment, a muscle cells are generated according to a method of the invention by introducing into the progenitor cells a constitutively active MEF2 polypeptide and a caspase inhibitor.

Further provided by the invention is an isolated progenitor cell which contains an exogenous nucleic acid molecule encoding a MEF2 polypeptide or an active fragment thereof. A progenitor cell of the invention can contain, for example, a nucleic acid molecule encoding a MEF2 polypeptide, or active fragment thereof, operatively linked to a heterologous regulatory element. In one embodiment, the MEF2 polypeptide is a human MEF2 polypeptide or an active fragment thereof. In another embodiment, the MEF2 polypeptide is a MEF2C polypeptide or an active fragment thereof. In further embodiments, the MEF2 polypeptide is a constitutively active MEF2 polypeptide, which can be, for example, a constitutively MEF2A, MEF2B, MEF2C or MEF2D polypeptide and further can be, for example, a constitutively active MEF2 polypeptide in which the native MEF2 activation domain is replaced with a heterologous activation domain. In yet a further embodiment, a constitutively active MEF2 polypeptide is resistant to caspase cleavage. A constitutively active MEF2 polypeptide useful in a progenitor cell of the invention can be, for example, a MEF2A/VP16, MEF2A/GAL4, MEF2B/VP16, MEF2B/GAL4,

MEF2C/VP16, MEF2C/GAL4, MEF2D/VP16 or MEF2D/GAL4 fusion protein. A constitutively active MEF2 polypeptide also can be a modified MEF2 polypeptide in which one or all of the p38 kinase phosphorylation sites in the MEF2 transactivation domain are substituted with an aspartic or glutamic acid residue.

A progenitor cell useful in the invention can be a human progenitor cell such as a human stem cell and, if desired, can be a CD133-positive human progenitor cell. Progenitor cells useful in the invention can be selected such that they are enriched for specific markers. Human progenitor cells useful in the invention include, for example, CD133-positive human progenitor cells; CD133-positive/CD34-positive human progenitor cells; CD133-positive/CD34-negative human progenitor cells; CD133-positive/CD34-negative/CD45-negative; CD34-negative/CD38-negative/Lin-negative human progenitor cells; and CD34-positive/CD38-negative/ Lin-negative/ Thy-1-negative human progenitor cells.

Progenitor cells useful in the invention include stem cells, which can be, for example, embryonic stem cells such as human embryonic stem cells. Progenitor cells useful in the invention also can be, for example, human hematopoietic progenitor cells including the most undifferentiated, pluripotent hematopoietic progenitor cells, which can be denoted "hematopoietic stem cells." In one embodiment, a progenitor cell of the invention is a human progenitor cell such as a CD133-positive/CD34-negative/Lin-negative human progenitor cell containing an exogenous nucleic acid molecule encoding a human MEF2C polypeptide or active

fragment thereof. In still further embodiments, a progenitor cell of the invention is a human progenitor cell such as a CD133-positive/CD34-negative/Lin-negative human progenitor cell that contains an exogenous nucleic acid molecule encoding a constitutively active MEF2 polypeptide or an active fragment thereof.

As used herein, the term "stem cell" means a pluripotent cell type which can differentiate under the appropriate conditions to give rise to all cellular lineages. Thus, a stem cell differentiates to neuronal cells, hematopoietic cells, muscle cells, adipose cells, germ cells and all other cellular lineages. A stem cell can be an embryonic stem cell. Where the term "hematopoietic stem cell" is used, it is understood that this term refers to cells that are committed to the hematopoietic lineage but which can differentiate to all cells of the hematopoietic lineage.

As used herein, the term "embryonic stem cell" is synonymous with "ES cell" and means a pluripotent cell type derived from an embryo which can differentiate to give rise to all cellular lineages. Examples of cell markers that indicate a human embryonic stem cell include the Oct-4 transcription factor, alkaline phosphatase, SSEA-4, TRA 1-60, and GCTM-2 epitope as described in Reubinoff et al., *supra,* 2000. Examples of cell markers that indicate a differentiated neuronal cell including neurofilament proteins, β-tubulin, Map2a+b, synaptophysin, glutamic acid decarboxylase, TuJ1, SNAP 25, transcription factor Brn-3, and $GABA_A$ α2 receptor subunit as described in Reubinoff et al., Nat. Biotech. 18:399-404 (2000); Ghosh and Greenberg, Neuron 15:89-103

(1995); Bain et al., Devel. Biol. 168:342-357 (1995); and Williams et al., Neuron 18:553-562 (1997).

The methods of the invention also can be used to differentiate progenitor cells in the same manner as the disclosed methods for differentiating stem cells. As used herein, the term "progenitor cells" means any cells that are capable of differentiating into the desired cell type such as a neuronal cell under the appropriate conditions. Progenitor cells can be multipotent or unipotent and can be stem cells, precursor cells, primary cells or established cells. Progenitor cells such as stem cells generally are distinct from neurons in that they lack neuronal markers such as the nuclear protein NeuN, neurofilament and microtubule-associated protein 2 (MAP2) as well as the neuronal-like processes characteristic of mature neurons. In one embodiment, progenitor cells are cells other than P19 embryonic carcinoma cells, which are cells from an established cell line that differentiates to neurons when treated with retinoic acid and to myocytes when treated with dimethylsulfoxide. In another embodiment, progenitor cells are primary cells, which is a well known term in the art for cells which are derived directly from an organism and which have limited growth capacity in culture.

A progenitor cell useful in the invention can be multipotent or unipotent. As used herein in reference to a progenitor cell, the term "multipotent" is synonymous with "pluripotent" and means a progenitor cell capable of differentiating into two or more distinct lineages, including the neuronal lineage. Multipotent

progenitor cells such as stem cells, which are generally nestin-positive cells, are distinguished from unipotent precursor cells, which are generally Hu-positive cells. Expression of nestin and Hu can be determined, for example, by immunocytochemistry as disclosed in Example II. A multipotent progenitor cell is capable of differentiating into at least three or more, four or more, or five or more distinct lineages, including the neuronal lineage.

The methods of the invention are useful for differentiating progenitor cells to produce a population containing protected neuronal cells. As used herein, the term "neuronal cell" means a nerve cell and is characterized, in part, by containing one or more markers of neuronal differentiation. Such a marker can be, for example, neurofilament, NeuN or MAP2. A neuronal cell further generally is characterized as containing neuronal-like processes as shown in *Figure 6F*.

The results disclosed herein indicate that the p38α/MEF2 cascade protects differentiating cells from death during neurogenesis. Thus, the methods of the invention rely, in part, on inducing the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (p38 MAP kinase/MEF2) pathway in a progenitor cell. The p38 MAP kinase/MEF2 pathway can be induced by any of a variety of means that result in an increase in MEF2A, MEF2B, MEF2C or MEF2D expression or activity. For example, a MEF2 polypeptide can be phosphorylated and activated, thereby inducing the p38 MAP kinase/MEF2 pathway. p38α, for example, is a known activator, and, therefore, transfection of a p38α encoding nucleic acid

molecule or treatment with an agent that increases p38α expression or activity can be used to induce the p38 MAP kinase/MEF2 pathway in a method of the invention (see Example VIII). Thus, transcription factors that increase transcription of a MEF2 polypeptide or p38α; kinases or other proteins that activate a MEF2 polypeptide; or upstream effectors such as PAK-γ can be used to induce the p38 MAP kinase/MEF2 pathway. One can readily assay for induction of the p38 MAP kinase/MEF2 pathway by assaying for MEF2 binding activity and transcriptional activity dependent on the presence of the MEF2 binding site.

Induction of the p38 MAP kinase/MEF2 pathway also can be achieved using a MEF2 activator, which is a small molecule that results in increased expression or activity of a MEF2 polypeptide or which is a mimetic or MEF2 function. A MEF2 activator can result in increased expression or activity of one or more MEF2 polypeptides, for example, may result in increased expression or activity of MEF2C without effecting expression or activity of MEF2A, MEF2B or MEF2D. Such a MEF2 activator can be an organic chemical, drug, nucleic acid molecule, peptide, peptidomimetic, polypeptide or other naturally or non-naturally occurring organic molecule, and can be, for example, a MEF2 mimetic. Exemplary MEF2 activators are transcription factors that upregulate MEF2 expression, molecules that compete for binding to a MEF2 inhibitor such as Cabin1 or histone deacetylase, and kinases that activate MEF2 polypeptides such as p38α. It is understood that a MEF2 activator can be useful in any of the methods of the invention in which the p38 mitogen-activated protein kinase/myocyte enhancer factor

2 (MEF2) pathway is induced. Thus, a MEF2 activator can be useful, for example, in differentiating progenitor cells to produce a cell population containing protected neuronal cells, in reducing the severity of a neurologic condition, in protecting a neuron or muscle cell from cell death, or in generating muscle cells.

MEF2 is normally sequestered in a transcriptionally inactive state by Cabin1 (Youn et al., Science 286:790793 (1999)). Thus, a MEF2 activator can be a factor that decreases expression of Cabin1 or a factor that promotes dissociation of MEF2 from Cabin1. Such a factor can be, for example, a fragment of Cabin1 or a fragment of MEF2 that competes for Cabin1 binding to MEF2, thereby dissociating MEF2 from Cabin1 and increasing the amount of active endogenous MEF2. Such MEF2 activators can be identified by preparing and screening fragments of Cabin1 and MEF2 using routine methods.

MEF2 also is post-translationally regulated by class II histone deacetylases, which bind the DNA-binding domain of MEF2 polypeptides (Lu et al., PNAS 97:4070-4075 (2000)). MEF2 activity can be maximally stimulated only when repression by histone deacetylases is relieved, for example, by calmodulin-dependent protein kinase signalling to the DNA-binding domain. Thus, a MEF2 activator can be a factor that decreases histone deacetylase expression or that promotes dissociation of MEF2 from histone deacetylase. Such a MEF2 activator can be, for example, a fragment of histone deacetylase or a fragment of MEF2 that competes for binding of MEF2 to histone deacetylase, thereby dissociating histone

deacetylase and increasing the amount of active MEF2 polypeptide. Such a MEF2 activator can be identified by preparing and screening fragments of histone deacetylase and MEF2 using routine methods.

An increase in either the p38α kinase or the big MAP kinase (Bmk1), also known as ERK5 kinase, increases MEF2 activity. Therefore, a MEF2 activator can be a molecule that increases the expression or activity of p38α kinase, for example, a nucleic acid molecule encoding p38α (Matsumoto et al., J. Biol. Chem. 274:13954-13960 (1999)). Similarly, a MEF2 activator also can be a molecule that increases the expression or activity of Bmk1/ERK5, for example, a nucleic acid molecule encoding Bmk1/ERK5 (English et al., Journal of Biological Chemistry 274:31588-31592 (1999); Kato et al., Nature 395:713-716 (1998)).

In one embodiment, the p38 MAP kinase/MEF2 pathway is induced by introducing a nucleic acid molecule encoding a MEF2 polypeptide into a progenitor cell under conditions suitable for expression of the MEF2 polypeptide in the cell. MEF2 polypeptides, which occur in a variety of isoforms and alternatively spliced forms, are characterized, in part, as belonging to the MADS-box family of transcriptional regulators. The MADS-box is a 57 amino acid motif located at the extreme N-terminus of MEF2 polypeptides (Figure 1A). This motif serves as a minimal DNA-binding domain and, in conjunction with an adjacent 29-amino acid extension designated the MEF2 domain, confers high-affinity DNA binding and dimerization (Molkentin et al., Mol. Cell. Biol. 16:2627-36 (1996). Within the MADS-box, MEF2

polypeptides share homology at several invariant residues with other members of the MADS-box family of transcription factors, including serum response factor (SRF). These conserved residues are important for DNA sequence recognition. While the MEF2 domain is unique to MEF2 factors, other MADS-box proteins contain domains with analogous functions. In addition to its role in DNA binding, the MADS-box mediates dimerization of MADS-box proteins, and the MEF2 domain is important for interactions with accessory factors. MEF2 polypeptides can homo- and heterodimerize but cannot interact with other MADS-box factors, indicating that specific residues within the MADS-box that establish the dimerization interface are not conserved outside the MEF2 family.

Vertebrate MEF2 polypeptides share about 50% amino acid identity overall and about 95% similarity throughout the highly conserved MADS-box and MEF2 domain, whereas they are divergent in their C-terminal regions. MEF2 polypeptides from invertebrates also are highly homologous to vertebrate MEF2 polypeptides in the MADS-box and MEF2 domain. The *Drosophila* MEF2 polypeptide, D-MEF2, binds the same DNA sequence as its vertebrate counterparts and can activate transcription through the MEF2 site in mammalian cells (Lilly et al., Proc. Natl. Acad. Sci. USA 91:5662-66 (1994) and Nguyen et al., Proc. Natl. Acad. Sci. USA 91:7520-24 (1994)).

MEF2 polypeptides, like other MADS-box proteins, bind an A/T-rich DNA sequence. The consensus MEF2 binding site is YTA(A/T)$_4$TAR. MEF2A, MEF2C and MEF2D have the same DNA binding specificity, whereas MEF2B binds the MEF2 consensus sequence with reduced

affinity compared to other family members. Nucleotides flanking the MEF2 site have been shown to profoundly influence DNA binding (Yu et al., Genes Dev. 6:1783-98 (1992); Andres et al., J. Biol. Chem. 270:23046-49 (1995); and Fickett Mol. Cell. Biol. 16:437-41 (1996)). Evidence suggests that the DNA binding site is bent upon high affinity DNA binding (Meierhans et al., Nucleic Acids Res. 25:4537-44 (1997)).

While the MADS-box and MEF2 domain are necessary and sufficient for DNA binding, they lack transcriptional activity on their own. The C-terminal regions of MEF2 polypeptides contain transcriptional activation domains and are subject to alternative splicing (Figure 1A), with some exons present ubiquitously and others muscle- or neural-specific. In MEF2A, an acidic exon with the sequence SEEEELEL (SEQ ID NO: 9) is specific to muscle and neural cells in which MEF2 DNA-binding activity is detected and is absent in MEF2 transcripts from a variety of cell types in which MEF2A protein is not detected (Yu et al., Genes Dev. 6:1783-98 (1992)). MEF2D contains a similar acidic exon (TEDHLDL; SEQ ID NO: 10), which is present only in transcripts from skeletal muscle, heart, and brain, and correlates with MEF2D-binding activity (Breitbart et al., Development 118:1095-106 (1993) and Martin et al., Mol. Cell. Biol. 14:1647-56 (1994)). The corresponding domain in MEF2C (SEDVDLLL; SEQ ID NO: 11) is present in transcripts from skeletal muscle only (McDermott et al., Mol. Cell. Biol. 13:2564-77 (1993)). Although the inclusion of these exons is not essential for DNA binding activity, their presence appears to correlate with high levels of MEF2 DNA-binding activity. There is relatively

little amino acid homology between the C-terminal regions of different MEF2 polypeptides, except for the short acidic exons described above and four serine/threonine-rich regions (Figure 1*B*).

As used herein, the term "MEF2 polypeptide" means a polypeptide that has MEF2 DNA binding activity in addition to activity as a transcriptional activator and includes polypeptides having substantially the amino acid sequence of MEF2A, MEF2B, MEF2C or MEF2D. Thus, a MEF2 polypeptide can have, for example, substantially the amino acid sequence of human MEF2A (SEQ ID NO: 2) shown in Figure 2, human MEF2B (SEQ ID NO: 4) shown in Figure 3; human MEF2C (SEQ ID NO: 6) shown in Figure 4, or human MEF2D (SEQ ID NO: 8) shown in Figure 5. A MEF2 polypeptide includes a MADS domain, a MEF2 domain and a transcriptional activation domain. It is understood that, while the MADS domain and MEF2 domains of a MEF2 polypeptide will be similar in structure to the MADS domain and MEF2 domain of a naturally occurring MEF2 polypeptide such as human MEF2C (SEQ ID NO: 6), the transcriptional activation domain of a MEF2 polypeptide may be structurally unrelated and can be, for example, a synthetic transcriptional activation or a heterologous transcriptional activation domain derived, for example, from VP16 or GAL4. One skilled in the art appreciates that a fragment of a MEF2 polypeptide that retains MEF2 DNA binding activity and transcriptional activity also can be useful in the methods and compositions of the invention.

The term MEF2 polypeptide encompasses a polypeptide having the sequence of a naturally occurring

human MEF2A polypeptide (SEQ ID NO:2), naturally occurring human MEF2B polypeptide (SEQ ID NO: 4), naturally occurring human MEF2C polypeptide (SEQ ID NO: 6) or naturally occurring human MEF2D polypeptide (SEQ ID NO: 8) and is intended to include related polypeptides having substantial amino acid sequence similarity to SEQ ID NOS: 2, 4, 6 or 8. Such related polypeptides typically exhibit greater sequence similarity to hMEF2A, hMEF2B, hMEF2C or hMEF2D than to other MADS box proteins such as serum response factor (SRF) and include species homologs such as primate, mouse, rat and *D. rerio* homologs, alternatively spliced forms, and isotype variants of the proteins shown in Figures 2 through 5.

As used herein, the term MEF2 polypeptide describes polypeptides generally including an amino acid region with greater than about 60% amino acid sequence identity in the combined MADS and MEF2 domains with hMEF2A (SEQ ID NO: 2), hMEF2B (SEQ ID NO: 4), hMEF2C (SEQ ID NO: 6) or hMEF2D (SEQ ID NO: 8). In particular, a MEF2 polypeptide can have greater than about 65% amino acid identity, preferably greater than about 70% amino acid identity, more preferably greater than about 75% amino acid identity, still more preferably greater than about 80% amino acid identity and most preferably greater than about 85%, 90% or 95% amino acid identity with the combined MADS and MEF2 domains of SEQ ID NOS: 2, 4, 6 or 8.

As used herein, the term "substantially the amino acid sequence," when used in reference to a MEF2 polypeptide or fragment thereof, is intended to mean a

polypeptide or fragment having an identical amino acid sequence, or a polypeptide, fragment or segment having a similar, non-identical sequence that is considered by those skilled in the art to be a functionally equivalent amino acid sequence. For example, polypeptide including substantially the same amino acid sequence as human MEF2C (SEQ ID NO: 6) can have an amino acid sequence identical to the sequence of human MEF2C (SEQ ID NO:6) shown in Figure 4, or a similar, non-identical sequence that is functionally equivalent. An amino acid sequence that is "substantially the amino acid sequence" can have one or more modifications such as amino acid additions or substitutions relative to the amino acid sequence shown, provided that the modified polypeptide retains the ability to bind the MEF2 binding site and to activate transcription.

Therefore, it is understood that limited modifications can be made without destroying the biological function of a MEF2 polypeptide or fragment useful in the invention. For example, minor modifications of hMEF2C (SEQ ID NO: 6) that do not destroy polypeptide activity also fall within the definition of a MEF2 polypeptide. Similarly, minor modifications of human MEF2A, -B, -C or -D that do not destroy polypeptide activity fall within the definition of a MEF2 polypeptide. Also, for example, genetically engineered fusion proteins that retain the DNA binding and transcriptional activation activity of a MEF2 polypeptide fall within the meaning of the term "MEF2 polypeptide" as used herein.

It is understood that minor modifications of primary amino acid sequence can result in polypeptides which have substantially equivalent or enhanced function as compared to the MEF2 polypeptides shown in Figures 2 through 5. These modifications can be deliberate, as through site-directed mutagenesis, or can be accidental such as through mutation in hosts harboring an encoding nucleic acid. All such modified polypeptides are included in the definition of a MEF2 polypeptide as long as MEF2 DNA binding activity and transcriptional activation activity are retained. Further, various molecules can be attached to a MEF2 polypeptide, for example, other polypeptides, carbohydrates, lipids, or chemical moieties. Such modifications are included within the definition of each of the polypeptides of the invention.

While native MEF2 polypeptides are activated through phosphorylation, for example, by p38 MAP kinase, constitutively active forms of MEF2 do not require such phosphorylation for activation. Any of the methods of the invention can be practiced using a constitutively active MEF2 polypeptide to induce the p38/MEF2 pathway.

As used herein in reference to a MEF2 polypeptide, the term "constitutively active" means a MEF2 polypeptide that has transactivation activity which is less dependent upon phosphorylation than the corresponding wild type MEF2 polypeptide. A constitutively active MEF2 polypeptide can have transactivation activity that is independent of phosphorylation. As disclosed herein, a MEF2 polypeptide can be cleaved by a caspase to produce a dominant

negative form of a MEF2 polypeptide having pro-apoptotic activity. In one embodiment, a constitutively active form of a MEF2 polypeptide is resistant to caspase cleavage.

A constitutively active MEF2 polypeptide can include, for example, a heterologous transactivation domain in addition to, or in place of, the native MEF2 transactivation domain. A constitutively active MEF2 polypeptide can be, for example, a MEF2A, MEF2B, MEF2C or MEF2D polypeptide containing a GAL4 or VP16 transactivation domain in addition to, or in place of, the native MEF2 transactivation domain. In specific embodiments, a constitutively active MEF2 polypeptide is a chimera in which the native MEF2 activation domain is replaced with a heterologous activation domain, for example, a constitutively active MEF2A/VP16, MEF2A/GAL4, MEF2B/VP16, MEF2B/GAL4, MEF2C/VP16, MEF2C/GAL4, MEF2D/VP16 or MEF2D/GAL4 fusion protein.

A constitutively active MEF2 polypeptide also can be a MEF2 polypeptide in which the native activation domain is modified such that transactivation does not depend on phosphorylation. A constitutively active MEF2 polypeptide can have, for example, one or modified phosphorylation sites within the transactivation domain, for example, one or more serine/threonine to aspartic acid/glutamic acid amino acid substitutions within the transactivation domain. See, for example, Watson et al., J. Neurosci. 18:751-762 (1998), which demonstrates that mutation of the Jun kinase phosphorylation site in c-Jun to aspartic acid produces a constitutively active c-Jun polypeptide that is independent of Jun kinase.

MEF2A is phosphorylated at Thr312, Thr319 and Ser453 within the transactivation domain, and MEF2C is phosphorylated at Thr293, Thr300 and Ser387 within the transactivation domain (Han et al., Nature 386:296-299 (1997); and Zhao et al., Mol. Cell. Biol. 19:21-30 (1999)). Thus, a constitutively active human MEF2A polypeptide can contain, for example, one or more amino acid substitutions such that one or all of Thr312, Thr319 and Ser453 are replaced with aspartic or glutamic acid. In addition, a constitutively active human MEF2C polypeptide can contain, for example, one or more amino acid substitutions such that one or all of Thr293, Thr300 and Ser387 are replaced with aspartic or glutamic acid. It is understood that analogous phosphorylation sites in MEF2B and MEF2D and other species homologs of MEF2A and MEF2C can be similarly modified to produce a constitutively active MEF2 polypeptide.

A variety of routine assays can be used to confirm constitutive activity of a MEF2 polypeptide including cotransfection assays using cerebrocorticol neurons, where constitutive activity is indicated by reporter activity significantly greater than wild type MEF2 transcriptional activity. For example, cerebrocorticol neurons can be cultured for about five hours using Lipofectamine2000 with the MEF2 expression vector and a luciferase reporter vector such as pGL2MEF2LUC, and luciferase activity determined by standard means.

As stated above, a MEF2 polypeptide can be cleaved by a caspase to produce a dominant negative form of a MEF2 polypeptide having pro-apoptotic activity.

Thus, in the presence of an activated caspase, protective MEF2 activity can be enhanced by a caspase inhibitor. Thus, in one embodiment of the invention, induction of the the p38 mitogen-activated protein kinase/myocyte enhancer factor 2 (MEF2) pathway is combined with treatment of the cell by a caspase inhibitor.

A variety of caspase inhibitors are useful in the invention including, for example, nucleic acids, polypeptides, peptides, peptidomimetics and non-peptide inhibitors such as small molecule drugs known in the art. As used herein, the term "caspase inhibitor" means any molecule that binds to and inhibits the activity of one or more caspases. Caspase inhibitors useful in the methods of the invention generally are cell permeable and have inhibitory activity *in vivo* and include viral and cellular gene products as well as synthetic inhibitors such as synthetic small molecules (Ekert et al., Cell Death and Differentiation 6:1081-1086 (1999)).

Such a caspase inhibitor can be a general, or non-selective, caspase inhibitor as well as a selective inhibitor. Selective inhibitors do not inhibit non-caspase cysteine proteases or serine proteases. Non-selective caspase inhibitors, which also inhibit one or more non-caspase protease inhibitors, include, for example, the cysteine protease inhibitor iodoacetamide. A caspase inhibitor also can be selective for one or more specific caspases. A caspase inhibitor can selectively inhibit caspase-3 or caspase-7 or a combination thereof and can be combined, for example, with a nucleic acid molecule encoding a MEF2C polypeptide, or an active fragment thereof. An exemplary caspase inhibitor which

is selective for caspases-3 and -7 is a non-peptide inhibitor such as a isatin sulfonamide (see, for example, Lee et al., J. Biol. Chem. 275:16007-16014 (2000)). A selective caspase inhibitor also can be selective for caspase-3, caspase-6, caspase-7 or caspase-8, or any combination thereof, and can be combined, for example, with a nucleic acid molecule encoding a MEF2A polypeptide, or an active fragment thereof.

A caspase inhibitor can be, for example, the cytokine response modifier A (CrmA) polypeptide, or an encoding nucleic acid molecule, which inhibits caspases -1 and -3; or the p35 baculovirus protein, or an encoding nucleic acid molecule, which inhibits caspases-1, -3, -6, -7, -8 and -10 but does not inhibit non-caspase cysteine proteases or serine proteases (Clem et al., Science 254:1388-1390 (1991)). A caspase inhibitor also can be an inhibitor of apoptosis protein (IAP) or an encoding nucleic acid molecule. IAPs useful as caspase inhibitors in a method of the invention include XIAP and Survivin, which inhibit caspases-3 and -7.

A caspase inhibitor also can be a synthetic caspase inhibitor such as a pseudosubstrate which acts as reversible or irreversible competitive inhibitors of caspases. Active site mimetic peptide ketones are useful, for example, as selective caspase inhibitors. Such caspase inhibitors include, for example, benzylcarbonyl (z)-VAD-fluoromethylketone (fmk), z-VAD-fmk/chloromethylketone (CMK), z-DEVD-fmk/cmk; and z-D-cmk. Additional caspase inhibitors include the halomethyl ketone-linked peptide YVAD, Ac-WEHD-CHO, Ac-DEVD-CHO, Ac-YVAD-CHO, t-butoxycarbonyl-IETD-CHO, and

t-butoxycarbonyl-AEVD-CHO (Ekert et al., *supra*, 1999). The skilled person understands that these and other caspase inhibitors can be useful in the invention. See, for example, Nicholson, Nature 407:310-316 (2000), WO 00/55114, and Garcia-Calvo et al., J. Biol. Chem. 273:32608-32613 (1998)).

As used herein in reference to a neuronal cell, the term "protected" means a cell that is induced to undergo neurogenesis and is more resistant to apoptotic cell death than a cell in which the p38 MAP kinase/MEF2 pathway is not induced, or is induced to a lesser extent. Thus, a population containing protected neuronal cells will exhibit less apoptosis than a population that does not contain "protected" neuronal cells.

The percentage of apoptotic cells in a population can be determined by a variety of assays well known in the art. Such methods include light microscopy for determining the presence of one or more morphological characteristics of apoptosis, such as condensed or rounded morphology, shrinking and blebbing of the cytoplasm, preservation of structure of cellular organelles including mitochondria, and condensation and margination of chromatin. The percentage of apoptotic cells also can be determined by assaying apoptotic activity using terminal deoxytransferase-mediated (TdT) dUTP biotin nick end-labeling (TUNEL) (Gavriel et al., J. Cell Biol. 119:493 (1992); Gorczyca et al., Int. J. Oncol. 1:639 (1992); Studzinski (Ed.), Cell Growth and Apoptosis, Oxford: Oxford University Press (1995)). ApopTag™ (ONCOR, Inc., Gaithersburg, MD) is a commercially available kit for identification of

apoptotic cells using digoxygenin labeling. In addition, apoptotic cells can be identified by detecting characteristic nucleosomal DNA fragments using agarose gel electrophoresis (Studzinski, *supra*, 1995; Gong et al., Anal. Biochem. 218:314 (1994)) or using DNA filter elution methodology to detect apoptosis-associated DNA fragmentation (Bertrand et al., Drug Devel. 34:138 (1995)). One skilled in the art understands that these, or other assays for apoptosis, can be performed using methodology routine in the art.

In the methods of the invention, progenitor cells are contacted with a differentiating agent. In one embodiment, the differentiating agent is retinoic acid, for example, all *trans*-retinoic acid. In another embodiment, the differentiating agent is neurotrophic factor 3, epidermal growth factor, insulin-like growth factor 1 or a platelet-derived growth factor.

As used herein, the term "differentiating agent" means a naturally occurring or synthetic cytokine, growth factor or other compound that causes or enhances a progenitor cell to have one or more characteristics of a neuronal cell. A differentiating agent useful in the invention can be, for example, retinoic acid such as all-*trans* retinoic acid; neurotrophic factor 3 (NT3); epidermal growth factor (EGF); insulin-like growth factor-1 (IGF-1); platelet derived growth factor (PDGF), or a combination of two or more of these factors. For example, EGF, IGF-1 and PDGF can be used together as a differentiating agent. Basic fibroblast growth factor (bFGF) or another factor that enhances proliferation of precursor cells can optionally be used prior to treating

with a differentiating agent such as EGF, IGF-1 and PDGF. One skilled in the art understands that one or more factors such as brain-derived neurotrophic factor (BDNF) also can be added to promote neuronal cell survival.

For use in the methods or compositions of the invention, embryonic stem cells can be obtained from a variety of mammals including, for example, mice, cows, primates and humans by methods well known in the art. For example, murine embryonic cells can be isolated from a mouse as described in Forrester et al., Proc. Natl. Acad. Sci. USA 88:7514-7517 (1991) or Bain et al., Devel. Biol. 168:342-357 (1995). Briefly, two-stage cell embryos can be isolated from fertilized female mice about 45 hours after injection with human chorionic gonadotropin. The two blastomeres can be fused by electrical impulse and cultured in M16 medium until the four cell stage is reached. ES cells can be grown on gelatin coated tissue culture flasks in DMEM (Dulbecco's modified Eagle's medium) containing high glucose and 1-glutamine (BRL) supplemented with 10% fetal bovine serum, 10% newborn calf serum, nucleosides stock, 1000 units/ml leukemia inhibitory factor, and 0.1 mM 2-mercaptoethanol.

Embryonic stem cells can be isolated from primates as described in Thomson (U.S. Pat # 5,843,780). Briefly, blastocysts can be removed from fertilized female monkeys 6-8 days after onset of ovulation, treated with pronase (Sigma) to remove the zona pellucida, rabbit anti-rhesus monkey spleen cell antiserum (for blastocysts from rhesus monkeys) and guinea pig complement (Gibco BRL), and washed in DMEM. The inner cell mass (ICM) can be removed from the lysed blastocyst with a pipette and

plated on mouse gamma radiation inactivated embryonic fibroblasts. After 7 to 21 days the ICM derived masses can be removed with a micropipette, treated with 0.05% trypsin-EDTA (Gibco BRL) and 1% chicken serum, and replated on embryonic feeder cells. Colonies demonstrating ES morphology, characterized by compact colonies with a high nucleus to cytoplasm ratio and prominent nucleoli, can then be split as described above. The ES cells can be split by trypsinization or exposure to Dulbeco's phosphate buffered saline containing 2 mM EDTA every 1-2 weeks when cultures become dense.



Embryonic stem-like cells also can be isolated from cows as described in Cibelli et al., Nat. Biotech. 16:642-646 (1998). Briefly, oocytes can be removed from freshly slaughtered cows and placed in maturation medium M199 (Gibco), 10% fetal calf serum (FCS), 5 ug/ml bovine leutinizing hormone (Nobl) and 10 ug/ml pen-strep (Sigma) for 22 hours at 38.5°C. Oocytes can then be fertilized *in vitro* and cultured on mouse embryonic fibroblast feeder layers and CR2 with 6 mg/ml BSA until they reach the blastocyst stage. ES cells can be isolated from the blastocyst by mechanical removal of the zona pellucida and trophoblast with a 22 gauge needle and placed under mouse embryonic fibroblast feeder layers for one week. A small colony of the resulting cell mass can be removed and cultured on top of gamma irradiation inactivated mouse embryonic fibroblast feeder layer as cultures become dense.

Embryonic stem cells can be isolated from human blastocysts as described in Reubinoff et al., Nat. Biotech. 18:399-404 (2000). Briefly, fertilized oocytes

can be cultured to the blastocyst stage and the zona pellucida digested by pronase (Sigma). The inner cell mass can be removed by immunosurgery with anti-human serum antibody (Sigma) and exposure to Guinea pig complement (BRL), and cultured on a mitomycin C mitotically inactivated mouse embryonic feeder cell layer in DMEM (BRL) supplemented with 20% fetal bovine serum (FBS, Hyclone) 0.1 mM 2-mercaptoethanol, 1% non essential amino acids, 2 mM glutamine, 50 units/ml penicillin and 50 ug/ml streptomycin (BRL)and 2,000 units/ml recombinant leukemia inhibitory factor. Cell mass clumps can be removed with a micropipette and replated on fresh feeder layer every six to eight days.

Human stem cells can be obtained, for example, from cord blood, which is highly enriched in primitive cells and contains a CD133-positive/ CD34-positive population. These cells can be efficiently isolated by methods well known in the art, for example, the Miltinyl MACS system. If desired, the CD133-positive/CD34-positive population can be expanded by culturing *in vitro* with Flt3L + TPO to produce as much as an 160-fold expansion in long-term culture potential and a $2x10^6$ fold expansion in the number of progenitor cells.

Human progenitor cells useful in the invention include human embryonic stem cells, human hematopoietic stem cells and other progenitor cells isolated from adult human blood or from cord blood of newborn infants. In one embodiment of the invention, the progenitor cell population is enriched in CD133 (AC133)-positive/CD34-positive progenitor cells. In a further embodiment of the invention, the progenitor cell population is enriched

in CD133-positive/CD34-negative progenitor cells. Such specific progenitor cell populations can be isolated, for example, with magnetic-activated cell sorting, fluorescence-activated cell sorting (FACS), or related methods well known in the art as described further below. It further is understood that *in vitro* expansion of progenitor or stem cells such as human progenitor or stem cells can be performed, if desired, with one or more of the following factors: SCF, IL-3, IL-6, flt3L, LIF, IL-11, TGF-β, TPO, EGF and bFGF, which are commercially available, for example, from Biosource (Camarillo, CA), R & D Systems (Minneapolis, MN) and Chemicon (Temecula, CA). Various protocols for expansion and useful concentrations of particular factors are well known in the art.

In one embodiment, human progenitor cells are obtained from peripheral blood. Donors can be treated with recombinant human G-CSF (rhG-CSF), such as Neupogen (Amgen; Thousand Oaks, CA), or recombinant human GM-CSF (rhGM-CSF), such as Leukine (Immunex; Seattle, WA), or both. In a further embodiment, the human progenitor cells are primitive cells characterized as CD34+, Thy-/dim, CD38-, which can be obtained, if desired, from G-CSF or GM-CSF treated to donors to increase long-term culture potential. In one embodiment, human progenitor cells are CD34+, Thy-/dim, CD38- cells obtained from donors treated with G-CSF in combination with GM-CSF.

Methods well known in the art can be used to collect progenitor cells from human peripheral blood or cord blood. Apheresis can be used to collect white blood cells, for example, four to five days following treatment

with G-CSF, GM-CSF or a combination of G-CSF and GM-CSF, generally yielding 4 x10$^6$ CD34-positive cells/kg of body weight.

A Ceprate SC immunoaffinity column commercially available from Cellpro (Bothell, WA) can be used to isolate a CD133-positive progenitor cell population for use in a method of the invention. The desired cell population binds the column matrix via a biotin conjugated antibody linked to the column matrix and is released by mechanical shaking. Ceprate SC immunoaffinity can be used to yield about 50% CD34-positive cells with about 16-99% purity.

CD133-positive human progenitor cells also can be isolated, for example, using an Isolex 300 magnetic cell separator (Baxter Healthcare Corporation; Deerfield, IL), which relies on mouse monoclonal IgG1 antibodies and magnetic beads coated with anti-mouse IgG1 antibody. Release of the progenitor cells by peptidase treatment yields about 50% CD34-positive cells with 33-100% purity.

Additional art-accepted procedures for isolation of human stem and progenitor cells include the magnetic activated cell sorting system (MACS) commercially available from Miltenyi Biotech (Auburn, CA). In this sorting system, small magnetic beads coated with secondary antibody are bound to the primary antibody-treated cells and retained on a ferromagnetic matrix column by a strong magnet. Cells are released by removal of the magnet to give greater than 50% recovery and greater than 90% purity of the desired cells.

Fluorescence-activated cell sorting (FACS) also is a well known method that can be used to isolate the desired progenitor or stem cell population. Using this methodology, cells are selected by attachment of fluorescent-conjugated antibodies to give greater than 90% purity of the recovered stem or progenitor cells.

If desired, isolated stem or progenitor cells can be assayed for the ability to repopulation bone marrow of a sublethally irradiated nonobese diabetic/severe combined immunodeficient (NOD-SCID) mouse, using methods well known in the art, as described, for example, in Miyoshi et al., Science 283:682-686 (1999).

In one embodiment, progenitor cells useful in the invention are human CD34-negative bone marrow cells such as CD133-positive/CD34-negative cells. In a further embodiment, the progenitor cells are CD34-negative/ Lin-negative cells. Such cells can have characteristics of stromal cells and are capable, for example, of repopulating the bone marrow of NOD/SCID mice following sublethal irradiation. In one embodiment, progenitor cells useful in the invention are CD133-positive/ CD34-negative/Lin-negative cells.

Methods of preparing progenitor or stem cell populations enriched for particular markers are well known in the art. For example, a CD133-positive/CD34-positive hematopoietic stem and progenitor cells can be prepared as set forth in Yin et al., Blood 90:5002-5012 (1997); CD133-positive/CD34-negative/CD45-negative progenitor cells can be prepared

as described, for example, in Uchida et al., Proc. Natl. Acad. Sci., USA 97:14720-14725 (2000). In addition, CD34-negative/CD38-negative/Lin-negative human hematopoietic stem cells and CD34-positive/CD38-negative/Lin-negative/Thy-1-negative hematopoietic stem cells can be prepared, for example, as described in Bhatia et al., Nature Medicine 4:1038-1045 (1998).

In the methods of the invention, progenitor or stem cells such as embryonic stem cells are contacted with a differentiating agent to induce differentiation of the cells along the neuronal pathway. Methods for differentiating embryonic stem cells by growth of the cells to high density are described in Reubinoff et al., Nat. Biotech. 18:399-404 (2000). Methods differentiating expanded CNS cells by initial growth in the presence of a mitogen such as basic fibroblast growth factor (bFGF) followed by removal of bFGF are described in Johe et al. Genes Develop. 10:3129-3140 (1996). Induction of neurogenesis by addition of growth factors can be achieved with platelet derived growth factor (PDGF) including for example PDGF-AA, PDGF-AB or PDGF-BB administered in the absence of bFGF as described in Johe et al., *supra*, 1996. Induction of neuronal differentiation can also be achieved *in vitro* by removal of fibroblast growth factor-2 and subsequent addition of insulin like growth factor-1, heparin or neurotrophin-3 as described in Brooker et al., J. Neurosci. Res. 59:332-341 (2000) and Ghosh and Greenberg, Neuron 15:89-103 (1995); addition of platelet-derived growth factor as described in Williams et al., Neuron 18:553-562 (1997); addition of insulin like growth factor-1 alone or in combination with brain derived neurotrophic factor as

described in Arsenijevic and Weiss, J Neurosci. 18:2118-2123 (1998); and exposure to retinoic acid as described in Bain et al. Devel. Biol. 168:342-357 (1995).

In a preferred embodiment, a nucleic acid molecule encoding a MEF2 polypeptide is introduced into a progenitor cell such as an embryonic stem cell. A variety of methods are known in the art for introducing a nucleic acid molecule into a progenitor cell such as an embryonic stem cell. Such methods include microinjection, electroporation, lipofection, calcium-phosphate mediated transfection, DEAE-Dextran-mediated transfection, polybrene- or polylysine-mediated transfection, and conjugation to an antibody, gramacidin S, artificial viral envelopes or other intracellular carriers such as TAT. For example, embryonic stem cells can be transformed by microinjection as described in Cibelli et al., Nat. Biotech. 16:642-646 (1998) or Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995); by lipofection as described in Choi (U.S. Pat # 6,069,010) or Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995); by electroporation as described in Current Protocols in Molecular Biology, John Wiley and Sons, pp 9.16.4-9.16.11 (2000) or Cibelli et al., Nat. Biotech. 16:642-646 (1998); or by fusion with yeast spheroplasts Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995). A MEF2 polypeptide also can be delivered to stem or progenitor cells as a TAT/MEF2 polypeptide fusion by techniques well known in the art as described in Nagahara et al., Nature Medicine 4:1449-1452 (1998).

Viral vectors can be particularly useful for introducing a nucleic acid molecule encoding a MEF2 polypeptide in a method of the invention; such vectors include, for example, retroviral vectors, lentiviral vectors, adenoviral vectors and adeno-associated vectors (AAV), herpesvirus vectors (see, for example, Kaplitt and Loewy, Viral Vectors: Gene Therapy and Neuroscience Applications Academic Press, San Diego, California (1995); Chang, Somatic Gene Therapy CRC Press, Boca Raton, Florida (1995)). Lentiviral, retroviral and adeno-associated vectors can be useful, for example, for permanent expression, and adenovirus and herpesvirus can be used to achieve transient expression lasting for several months to about one year. It is understood that both permanent and transient expression can be useful in a method of the invention and in producing a stem or progenitor cell of the invention.

It is understood by those skilled in the art of gene therapy that a progenitor cell also can be engineered to express one or more gene products that are therapeutically useful. For example, for treatment of Parkinson's disease, a progenitor cell such as an embryonic stem cell can express, for example, the catecholamine enzyme tyrosine hydroxylase, thereby increasing dopamine-ß-hydroxylase activity upon intracerebral grafting (Jiao et al., Nature 362:450 (1993); see, also, Dhawan et al., Science 254: 1509 (1991); and Barr and Leiden, Science 254:1507 (1991)). Similarly, for treatment of Alzheimer's disease, a progenitor cell can express a nucleic acid molecule encoding nerve growth factor, thereby promoting cell survival of the cholinergic neurons that are typically

lost in Alzheimer's disease (Rosenberg et al., Science 242:1575-1578 (1988)). In a similar manner, a progenitor cell can express encephalin for treatment of neuropathic disorders involving intractable pain. One skilled in the art recognizes that these and other combinations are encompassed by the methods of the invention for differentiating progenitor cells to produce a population containing protected neuronal cells.

A progenitor cell such as an ES cell further can be engineered to express one or more anti-apoptotic gene products such as a member of the Bcl-2 family, for example, Bcl-2 (Anderson, Trends Pharm. Sci. 18:51 (1997) or Bcl-$X_L$; and Gross and et al., Genes Dev. 13:1899-1911 (1999)), or a member of the inhibitor of apoptosis (IAP) family such as c-IAP-1, c-IAP-2, XIAP or NIAP (Deveraux and Reed, Genes Dev. 13:239-252 (1999)). A progenitor cell further can be optionally engineered to express a basic helix-loop-helix protein (bHLH), especially a bHLH protein naturally expressed in neuronal cells such as Mash-1, which can functionally interact with a MEF2 polypeptide (Mao and Nadal-Ginard, J. Biol. Chem. 271:14371-14375 (1996); Black et al., J. Biol. Chem. 271:26659-26663 (1996). A progenitor cell such as an ES cell also can be engineered to express one or more factors that promote differentiation including, for example, neuroD, neuroD2, neuroD3, neurogenin1, neurogenin2, neurogenin3, MATH1 or MATH2 (Lee, Curr. Opin. Neurobiol. 7:13-20 (1997)). Such a factor can be expressed instead of or in addition to application of the differentiating agent to the progenitor cell.

Previous methods of producing neuronal cells have suffered from the shortcoming that the populations produced are heterogenous and contain relatively few neurons. A method of the invention is advantageous in that it can be used to produce a population containing protected neuronal cells containing a large proportion of neuronal cells, for example, at least 50% neuronal cells. In other embodiments, the population produced includes at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more neuronal cells. The proportion of neuronal cells can be determined by assaying for one or more characteristic neuronal markers such as the presence of NeuN, neurofilament or MAP2.

A method of the invention for differentiating progenitor cells to produce a population containing protected neuronal cells can optionally include the step of transplanting into a patient cells treated to induce the p38/MEF2 pathway. In a method of the invention, cells can be transplanted, for example, into the brain, eye (retina) or spinal cord after neuronal injury or damage. Thus, cells treated to induce the p38/MEF2 pathway can be transplanted into a patient having or at risk of, for example, stroke or a neurodegenerative disease such as Alzheimer's disease; Huntington's disease; amyotrophic lateral sclerosis; Parkinson's disease; epilepsy; brain or spinal cord trauma; multiple sclerosis; optic neuropathy such as glaucoma; infection of the central nervous system; multiple system atrophy affecting the brain; or another acute or chronic neurodegenerative condition. Upon transplantation, the cells begin to differentiate or continue differentiating

to produce a cell population containing protected neuronal cells.

As used herein, the term "patient" means any animal containing neurons, for example, a mammal such as a mouse, rat, dog, primate or human. A patient typically suffers from or is at high risk of developing a neurodegenerative disorder such as Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis or multiple sclerosis; hypoxia-ischemia (stroke); epilepsy; head or spinal cord injury; optic neuropathies including glaucoma and macular degeneration, and disorders of photoreceptor degeneration such as retinitis pigmentosa; metabolic, mitochondrial or infectious brain abnormalities such as encephalitis, or suffers from neuropathic pain (see, for example, Lipton and Rosenberg, New Engl. J. Med. 330: 613 (1994)).

Cells can be transplanted into a patient, for example, into the brain or spinal cord using well known methods for transplanting or "grafting" neurons as described, for example, in McDonald et al., Nat. Med. 5:1410-1412 (1999), and summarized in Dunnett et al., Brit. Med. Bulletin 53:757-776 (1997). Methods for preventing or ameliorating rejection, for example, using cyclosporinA treatment, also are known in the art.

Those skilled in the art understand that the steps of contacting the progenitor cells with a differentiating agent and inducing the p38 MAP kinase/MEF pathway can be performed in any order or simultaneously. It further is understood that a progenitor population in which the p38 MAP kinase/MEF2 pathway has been induced

can be transplanted into a patient prior to, during or after differentiation of the progenitor cells into neuronal cells. In one embodiment, cells are transplanted prior to or during differentiation. Where cells are transplanted prior to differentiation, the neuronal environment can drive the cells into the desired neuronal cell type, rather than, for example, muscle cells due to the presence of the appropriate environmental cues. In view of the above, it is clear that differentiation can occur *in vitro* or *in vivo*, or can occur partially *in vitro* and partially *in vivo*.

The invention further provides a pharmaceutical composition containing a MEF2 mimetic, which is a peptide or non-peptide molecule that mimics MEF2 function. The invention further provides a pharmaceutical composition containing a p38 mimetic, which is a peptide or non-peptide molecule that mimics p38 function.

Further provided by the invention is a pharmaceutical composition containing a MEF2 activator and a caspase inhibitor. MEF2 activators have been described herein above and include small molecules that result in increased expression or activity of a MEF2 polypeptide or that mimic MEF2 function. A MEF2 activator can be an organic chemical, drug, nucleic acid molecule, peptide, peptidomimetic, polypeptide or other naturally or non-naturally occurring organic molecule, and can be, for example, a MEF2 mimetic. Exemplary MEF2 activators are transcription factors that upregulate MEF2 expression, molecules that compete for binding to a MEF2 inhibitor such as Cabin1 or histone deacetylase, and kinases that activate MEF2 polypeptides such as p38α.

In one embodiment, the invention provides a pharmaceutical composition containing a nucleic acid molecule encoding a constitutively active MEF2 polypeptide, and a caspase inhibitor. Constitutively active MEF2 polypeptides have been described herein and can be prepared by methods well known in the art. A caspase inhibitor useful in a pharmaceutical composition of the invention can be any of the caspase inhibitors described above or another inhibitor well known in the art and can be, for example, a selective caspase inhibitor.

The present invention also provides a method of identifying a protective or differentiation gene, which can be, for example, a neuroprotective gene or a gene that contributes to neuronal or muscle cell differentiation. A method of the invention includes the steps of isolating a first cell population; isolating a second cell population, wherein the second cell population has an altered level or activity of a MEF2 polypeptide as compared to the first cell population; and assaying for differential gene expression in the first cell population as compared to the second cell population, whereby a gene differentially expressed in the second cell population as compared to the first cell population is identified as a protective or differentiation gene. In one embodiment, the first cell population is a progenitor cell population, the second cell population is a neuronal cell population, and the differentially expressed gene is a neuronal differentiation gene. In a further embodiment, the first cell population is a progenitor cell population, the second cell population is a muscle cell population, and

the differentially expressed gene is a muscle differentiation gene. In yet another embodiment, both cell populations are neuronal cell populations, the second cell population has been subject to a neuronal stress as compared to the first cell population, and the differentially expressed gene is a neuroprotective gene.

It is understood that the term "cell population" can mean a single cell or a collection of cells. In one embodiment, the first and second cell populations each are single cells. Where the cell populations are made up of more than a single cell, the cell populations can be homogeneous or heterogeneous. Furthermore, where the cell populations are made up of more than a single cell, it is understood that, while the second cell population as a whole has an altered level or activity of a MEF2 polypeptide as compared to the first cell population as a whole, there may or may not be an altered level or activity when individual cells from the first and second cell populations are compared.

An altered level of expression or activity of a MEF2 polypeptide can be achieved, for example, by comparing a particular tissue or cell of interest from a MEF2 knockout or conditional knockout mouse with a wild-type littermate. Cells or tissue samples can be obtained, for example, from the cerebrocortex or hippocampus of the brain or from cardiac muscle.

Further provided by the invention is a method of identifying a protective gene *in vitro*. The method is practiced by inducing the p38/MEF2 pathway in a cell *in vitro* to produce a protected cell; stressing the cell;

and assaying for differential gene expression in the protected cell as compared to gene expression in a control cell, whereby a gene differentially expressed in the protected cell as compared to the control cell is identified as a protective gene. In such a method of the invention, the p38/MEF2 pathway can be induced, for example, by introducing into the cell a nucleic acid molecule encoding a MEF2 polypeptide. The MEF2 polypeptide can be, for example, a human MEF2 polypeptide and further can be, if desired, a constitutively active MEF2 polypeptide. In one embodiment, a neuroprotective gene is identified by inducing the p38/MEF2 pathway in a neuron. In another embodiment, a muscle protective gene is identified by inducing the p38/MEF2 pathway in a muscle cell. In a method of the invention, the differential gene expression that identifies the protective gene can be increased or decreased gene expression.

The invention additionally provides a method of identifying a differentiation gene *in vitro* by inducing the p38/MEF2 pathway in a progenitor cell *in vitro* to produce a differentiated cell; and assaying for differential gene expression in the differentiated cell as compared to gene expression in a control cell, whereby a gene differentially expressed in the differentiated cell as compared to the control cell is identified as a differentiation gene. In a method of the invention, the p38/MEF2 pathway can be induced, for example, by introducing into the progenitor cell a nucleic acid molecule encoding a MEF2 polypeptide. The MEF2 polypeptide can be, for example, a human MEF2 polypeptide or a constitutively active MEF2 polypeptide.

In one embodiment, the differentiated cell is a neuronal cell, and, in a further embodiment, the differentiated cell is a muscle cell. The differential gene expression which serves to identify the differentiation gene can be increased or decreased gene expression.

A variety of means are well known in the art for assaying for differential gene expression. Such means include, for example, differential display such as mRNA differential display and differential display RT-PCR (DDRT-PCR); RNA fingerprinting; subtractive hybridization approaches and microarrays such as DNA microarrays. Differential display analysis can be used in a method of the invention, for example, as described in Jo et al., Methods Enzymol. 332:233-244 (2001); Staege et al., Immunogenetics 53:105-113 (2001); Fujimoto et al.., Hepatol. Res. 20:207-215 (2001). In addition, suppressive subtractive hybridization can be used to assay for differential gene expression in a method of the invention, for example, as described in Robert et al., Biol. Reprod. 64:1812-1820 (2001). Microarrays such as high-density oligonucleotide arrays and cDNA microarrays also can be useful for assaying for differential gene expression in a method of the invention and are well known in the art (see, for example, Lee et al., Science 285:1390-1393 (1999); Zirlinger et al., Proc. Natl. Acad. Sci., USA 98:5270-5275 (2001); Tsunoda et al., Anticancer Res. 21:137-143 (2001) and Khanna et al., Cancer Res. 61:3750-3759 (2001)). One skilled in the art understands that these and other methods for assaying for differential gene expression can be used in a method of the invention.

The following examples are intended to illustrate but not limit the present invention.

## EXAMPLE I

## INDUCTION OF MEF2 EXPRESSION IN P19 CELLS

This example demonstrates that retinoic acid induces MEF2 protein expression in P19 embryonic carcinoma cells.

P19 embryonal carcinoma cells terminally differentiate into neuronal cells after retinoic acid treatment, and the process of neurogenesis in P19 cells is similar to that of the mammalian central nervous system (McBurney, Int. J. Dev. Biol. 37:135-140 (1993) and Bain et al., Bioessays 16:343-348 (1994)). Moreover, the apoptotic cell death observed in neuronally-differentiating P19 cells parallels that seen in the fetal brain (Slack et al., J. Cell Biol. 129:779-788 (1995); Mukasa et al., Biochem. Biophys. Res. Commun. 232:192-197 (1997); Blaschke et al., Development 122:1165-1174 (1996); Jacks et al., Nature 359:295-300 (1992); and Kuida et al., Nature 384:368-372 (1996)).

MEF2, especially MEF2C, is expressed during neurogenesis in the rodent cerebral cortex (Leifer et al., Proc. Natl. Acad. Sci. USA 90:1546-1550 (1993)). To examine if MEF2 proteins are expressed during neurogenesis of P19 cells, gel shift assays were performed using the MEF2 binding site as a probe. While only very faint binding activity was detected in undifferentiated P19 cells, binding activity increased to high levels two days after retinoic acid treatment

(Figure 6*A*, lanes 1 and 2). Unlabeled MEF2 oligonucleotide abrogated the binding activity, indicating the specificity of the binding to the MEF2 site (Figure 6*A*, lanes 3 and 4). Although anti-MEF2A antibody did not affect the formation of the complex (Figure 6*B*, lanes 5 and 6), anti-MEF2C and anti-MEF2D antibodies supershifted the bands (Figure 6*B*, lanes 7 and 8), indicating the presence of MEF2C and MEF2D proteins in the complex. Immunoblotting revealed that the level of MEF2C and MEF2D proteins increased after retinoic acid treatment (Figure 6*C*). These results indicate that retinoic acid treatment induces MEF2 site-binding activity by MEF2C and MEF2D and an increase in MEF2C and MEF2D protein expression during neurogenesis of P19 cells.

Neuronal differentiation of P19 cells was induced as follows. P19 cells were purchased from the ATCC (CRL 1825) and maintained in a modified Eagle's minimum essential medium (MEM; Sigma, St. Louis, Missouri), supplemented with 10% heat inactivated fetal bovine serum (Intergen Co., Purchase, New York). For neuronal differentiation, 1 x $10^6$ P19 cells were cultured in a 10 cm diameter tissue culture dish with 300 pM 13-*cis* retinoic acid (Eastman Kodak, Rochester, New York) for two days. After trypsinization, the cells were again exposed to 300 pM retinoic acid and re-seeded onto a bacterial grade Petri dish to allow the cells to aggregate, thereby facilitating neuronal differentiation. After a one day incubation, cell aggregates were collected and dissociated with trypsin-EDTA. The dissociated cells were plated onto a tissue culture chamber slide (Nunc, Rochester, New York). The medium

was changed the day after plating and every two days thereafter.

Gel shift assays were performed as follows. Nuclear extracts of undifferentiated or retinoic acid-treated P19 cells were prepared as previously described (Okamoto et al., Brain. Res. Mol. Brain. Res. 74:44-54 (1999)). Protein concentrations were measured with a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, Illinois) using albumin as the standard. Nuclear extracts (5 µg/20 µl) were preincubated on ice for 10 minutes in a solution containing 20 mM Tris (pH 7.6), 10% glycerol, 1 mM dithiothreitol, 80 mM KCl, and 1 µg poly(dI-dC)•(dI-dC). $^{32}$P-end-labeled double stranded oligonucleotide representing the MEF2 binding site (TGGGCTATAAATAGCCGC; SEQ ID NO: 12) of the brain-specific creatine kinase gene was then added and incubated at room temperature for 20 minutes. The binding mixture was then electrophoresed on a 6% nondenaturing acrylamide gel in 0.25 x TBE for 1.5 h at 150 V. For supershift assays, antibodies against MEF2A (Santa Cruz Biotechnology, Inc., Santa Cruz, California), MEF2C (Leifer et al., Proc. Natl. Acad. Sci., USA 90:1546-1550 (1993)), or MEF2D (provided by Dr. B. Kosofsky, Massachusetts General Hospital, Boston) were added to the preincubation binding mixtures.

Immunoblotting was performed as follows. Whole cell lysates were prepared in RIPA buffer containing 0.1 mg/ml PMSF and 1 mM sodium vanadate. Proteins in 50 µg aliquots were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane (Amersham Life Science, Piscataway, New Jersey). Membranes were incubated

overnight at 4°C with primary antibody to MEF2C (1:1000), MEF2D (1:500), phospho p38 (1:1000, New England Biolabs, Inc., Beverly, Massachusetts), or p38α (1:1000, Santa Cruz Biotech). Horseradish peroxidase-linked anti-rabbit IgG (Vector) was used as the secondary antibody. Immunoblots were visualized with an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Piscataway, New Jersey).

**EXAMPLE II**

**OVEREXPRESSION OF MEF2C IN P19 CELLS**

These results indicate that stable overexpression of MEF2C transforms p19 cells into a mixed neurogenic/myogenic phenotype expressing neurofilament as well as the myosin heavy chain.

P19 cells exposed to DMSO develop into myogenic cells while P19 cells exposed to retinoic acid develop along a neurogenic pathway (McBurney, M. W., Int. J. Dev. Biol. 37:135-140 (1993)). To examine the potential role of MEF2C in the differentiation process, MEF2C was overexpressed in stable transformants of undifferentiated P19 cells in the absence of retinoic acid or DMSO. Undifferentiated P19 cells lacked immunoreactivity with MEF2C and also lacked neurofilament (Figure 6*E*). MEF2C-transfected cells expressed MEF2C protein in the nucleus, as determined by specific antibody labeling, and were neuronal in character as evidenced by labeling with anti-neurofilament. All transfected cells (from over 200 such cells scored) expressed MEF2C, stained with anti-neurofilament, and extended two neuronal-like processes, producing a bipolar appearance (Figure 6*F*).

None of the MEF2C-transfected cells stained positively for glial fibrillary acidic protein (GFAP), indicating that they did not manifest an astrocytic phenotype.

Overexpression of MEF2A has been shown to initiate the myogenic phenotype in the 10T1/2 fibroblast cell line (Kaushal et al., Science 266:1236-1240 (1994)) when co-expressed with other factors (MyoD or myogenin) (Molkentin et al., Cell 83:1125-1136 (1995)). Accordingly, P19 cells transfected with MEF2C were assayed for myogenic features by staining transfected cells with anti-myosin heavy chain antibody. All cells expressing MEF2C (over 200 counted) were also positive for myosin heavy chain label (Figure 6G). Taken together, these results indicate that transfection of undifferentiated P19 cells with MEF2C induces a bipolar cell phenotype that expresses both neuronal (neurofilament) and myogenic (myosin heavy chain) markers.

MEF2C was overexpressed in P19 cells essentially as follows. The phosphoglycerate kinase gene promoter-driven expression vector (pGK) was kindly provided by Dr. M. W. McBurney (University of Ottawa, Canada). Human MEF2C cDNA was inserted between the BamHI and XhoI sites of pGK to produce the expression vector pGK-MEF2C. $2 \times 10^5$ P19 cells were plated in a 6 cm diameter dish 24 h prior to transfection. Subsequently, 25 µg of the MEF2C expression vector (pGK-MEF2C) and 1 µg of the neomycin resistance gene expression vector (pSV neo) were co-transfected by calcium phosphate precipitation. The cells were washed 16 hours post-transfection and cultured in MEM with 10% serum.

After 24 hours, the cells were trypsinized and seeded onto a tissue culture chamber slide. The cells were maintained in 200 µg/ml Geneticin for 5 days to select the transfected cells.

Immunocytochemistry was performed as follows. Cultures were fixed with 3% paraformaldehyde at room temperature for 40 minutes. After washing three times with PBS, cells were permeabilized with 0.3% Triton X-100 for 5 minutes. The free aldehyde groups formed during the fixation were reduced by incubation with 1 mg/ml sodium borohydride three times for 5 minutes each. Cells were then washed three times in PBS. The fixed cells were incubated at 4°C overnight with primary monoclonal antibodies to microtubule-associated protein 2 (MAP2, 1:500; Sigma, clone HM-2), neurofilament H (1:250; Sternberger Monoclonals Inc., Lutherville, Maryland, SMI311), myosin heavy chain (1:250; Developmental Studies Hybridoma Bank, University of Iowa, Iowa, MF20), glial fibrillary acidic protein (GFAP, 1:400; Sigma, clone G-A-5), or rabbit antiserum to MEF2C (1:250) (Leifer et al., *supra,* 1993). Cells were then washed three times in PBS containing 0.2% Tween 20, and rhodamine-conjugated anti-mouse IgG or fluorescein-conjugated anti-rabbit IgG (each at 1:100; Boehringer Mannheim, Indianapolis, Indiana) was added as the secondary antibody. After a one hour incubation at room temperature, the cells were washed again and mounted. For Hu- and nestin-staining, cells were fixed with acid ethanol (95% ethanol: 5% acetic acid) for 30 minutes at room temperature. The fixed cells were then washed three times with PBS and incubated with a monoclonal antibody to Hu (1:200, gift of Drs. M.F. Marusich and J.A. Weston, University of

Oregon, OR) or to nestin (1:20, Developmental Studies Hybridoma Bank, Rat-401). After overnight incubation at 4°C, the samples were further washed and incubated with anti-mouse immunoglobulins conjugated to horseradish peroxidase (1:100; DAKO Corp., Carpinteria, California). A peroxidase reaction was performed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Stained preparations were examined under epifluorescence microscopy.

## EXAMPLE III

## CHARACTERIZATION OF P19 CLONES STABLY EXPRESSING A DOMINANT NEGATIVE FORM OF MEF2

This example describes the characterization of P19 clones in which MEF2C function is inhibited.

The role of endogenous MEF2 proteins in retinoic acid-induced neuronal differentiation of P19 was analyzed using a dominant negative form of MEF2. MEF2 proteins are functionally divided into two regions. The N-terminal region (containing the MADS and MEF2 domains) is responsible for specific DNA binding activity, while the C-terminal region is necessary for transcriptional activity (Martin et al., Mol. Cell. Biol. 14:1647-1656 (1994), and Molkentin et al., Mol. Cell. Biol. 16:2627-2636 (1996)). Since the MADS and MEF2 domains alone lack transcriptional activity, the N-terminal region of MEF2 acts as a dominant negative construct (Martin et al., Mol. Cell. Biol. 14:1647-1656 (1994)). Dominant negative MEF2 has been shown to inhibit myotube formation in myoblastic cell lines (Ornatsky et al., J. Biol. Chem. 272:33271-33278 (1997)).

Stable transformants of P19 cells were established which expressed the dominant negative N-terminus of MEF2C (residues 1 to 105). Three "vector-alone" transfected control clones were designated clones 2-1, 2-2 and 2-5; three clones expressing the MEF2 dominant negative were designated clones 2-7, 2-8 and 2-9. As a further control, two additional clones expressing a mutated form of the MEF2 dominant negative construct were produced and designated clones 2-16 and 2-21. Expression of the MEF2 dominant negative was monitored with gel shift assays. Binding activity of the MEF2 dominant negative was detected for clones 2-7, 2-8 and 2-9, but not for control clones 2-1, 2-2, 2-5, 2-16, or 2-21 (Figure 7A and data not shown). All transformants were morphologically indistinguishable from parent P19 cells.

The MEF2C dominant negative construct and a control were prepared as follows. Dominant negative MEF2C (amino acids 1-105) tagged with a flag sequence (pcDNAI-MEF2C 1-105 flag) and constitutively active MEF2C (pcDNAI-MEF2C 1-117/VP16) were obtained from Dr. Eric N. Olson (Southwestern Medical Center, Dallas). MEF2C 1-105 flag acts as a MEF2 dominant negative by binding to the MEF2 site without producing activation since it lacks the transactivation domain (Molkentin et al., Mol. Cell. Biol. 16:2627-2636 (1996)). MEF2C 1-105 flag cDNA was ligated into the BamHI/XhoI sites of pGK to produce pGK-DN. A mutation was engineered in the MEF2 dominant negative by changing the arginine residue at position 24 to a leucine, and this plasmid was designated pGK-DNmt. The mutated MEF2 dominant negative was unable to bind to the MEF2 site and therefore served as control to rule out

the possibility that the MEF2 dominant negative was affecting cell survival nonspecifically by binding to sites other than MEF2.

Stable transfection of P19 cells with a dominant negative form of MEF2 was performed as follows. Using the calcium phosphate precipitation method, 2 x 10 P19 cells were transfected with 24 µg of an empty expression vector (pGK), an expression vector encoding a dominant negative MEF2 (pGK-DN), or a mutated dominant negative MEF2 construct (pGK-DNmt) in addition to the neomycin resistance gene (pSV neo). The transfected cells were selected by exposure for 10 days to 200 µg/ml Geneticin. The selection medium was changed every two days. Stable clones expressing the MEF2 dominant negative and the mutated MEF2 dominant negative were selected with the reverse transcriptase-polymerase chain reaction.

**EXAMPLE IV**

**INHIBITION OF MEF2 FUNCTION DIMINISHES THE NUMBER OF NEURONAL CELLS**

This example demonstrates that the number of neuronal cells formed upon treatment of P19 cells with a neuronal differentiation stimulus is reduced when MEF2C function is inhibited.

Neuronal differentiation occurs via multiple sequential steps (Stemple and Mahanthappa, Neuron 18:1-4 (1997)). Nerve cells differentiate from unipotent progenitors, which arise from multipotent precursor cells. The effect of the MEF2 dominant negative on these

steps of neuronal differentiation was monitored by analyzing the appearance of differentiated neurons using antibodies to neuronal markers, such as neurofilament and MAP2. Stable transformants expressing a dominant negative form of MEF2C which inhibits MEF2 function were treated with retinoic acid to induce neuronal differentiation. After seven days, cells were fixed and stained with anti-MAP2 antibody. Although many MAP2-positive cells appeared in the control cultures (Figure 7*B*), the number of MAP2-positive cells was dramatically reduced in the MEF2 dominant negative cultures (Figure 7*C*). The difference between the number of MAP2-positive cells in the MEF2 dominant negative cultures and the control cultures was statistically significant (Figure 7*D*).

**EXAMPLE V**

**INHIBITION OF MEF2 FUNCTION REDUCES THE NUMBER OF PROGENITOR CELLS**

This example demonstrates that the number of progenitor cells is reduced by inhibition of MEF2c function with a dominant negative construct.

The number of precursor cells (multipotent precursor or unipotent precursor cells) was analyzed following inhibition of MEF2 function. The appearance of multipotent progenitors (nestin-positive cells) and unipotent precursors (Hu-positive cells) was monitored in control (clone 2-1) and MEF2 dominant negative cultures (clone 2-7). Nestin-positive and Hu-positive cells were counted before and after retinoic acid treatment for 3.0 days and 3.5 days, respectively. At these time points,

multipotent precursor cells (3.0 days) followed by unipotent precursor cells (3.5 days) appear in these cultures prior to the neuronally differentiated state (McBurney, M. W., Int. J. Dev. Biol. 37:135-140 (1993)). Hu-positive and nestin-positive cells were induced after retinoic acid treatment in both control (clone 2-1) and MEF2 dominant negative cultures (clone 2-7; Figure 7*A* and *C*). However, the number of Hu-positive and nestin-positive cells in the MEF2 dominant negative cultures was significantly smaller than than the number of Hu-positive and nestin-positive cells in control cultures (Figure 7*B* and *D*). These results indicate that interference with MEF2 activity can reduce the number of multipotent precursor and unipotent progenitor cells.

**EXAMPLE VI**

**INHIBITION OF MEF2 FUNCTION ENHANCES APOPTOTIC CELL DEATH DURING NEURONAL DIFFERENTIATION**

This example demonstrates that apoptotic cell death increases during neuronal differentiation by inhibition of MEF2C function.

Apoptotic cell death of differentiating cells is found widely in the developing fetal brain (Blaschke et al., Development 122:1165-1174 (1996)), and apoptosis is also observed during the course of neuronal differentiation of P19 cells (Slack et al., J. Cell Biol. 129:779-788 (1995), and Mukasa et al., Biochem. Biophys. Res. Commun. 232:192-197 (1997)). To examine the number of multipotent and unipotent precursor cells following interference with MEF2 cells, nuclear morphology was analyzed with the DNA dye Hoechst 33342 (Figure 9*A*) to

score the number of apoptotic cells after three days of retinoic acid treatment, at which time multipotent precursors were detected. Prior to the addition of retinoic acid, fewer than 1% of the cells exhibited apoptotic nuclei in control or MEF2 dominant negative cultures. After three days of retinoic acid treatment, a significant number of cells displayed apoptotic nuclei in the controls (transfected with either empty vector or the mutated form of the MEF2 dominant negative; Figure 9*B* and *C*). However, apoptosis was increased in the MEF2 dominant negative cultures (Figure 9*B*). These findings were confirmed by another apoptosis assay using the TUNEL technique (Figure 9*D*). Additionally, the number of apoptotic cells was increased in the MEF2 dominant negative cultures after 3.5 days of retinoic acid treatment, when unipotent precursor cells predominated. These results indicate that interference with MEF2 activity can increase apoptotic cell death during neuronal differentiation of P19 cells and can lead to a reduction in the number of multipotent and unipotent precursor cells. These results also indicate that MEF2 transcriptional activity can be essential for prevention of cell death during neuronal development.

Apoptotic assays were performed as follows. Cells were incubated with the DNA dye Hoechst 33342 (1 µg/ml) for 5 minutes at 37°C to observe nuclear morphology. After washing with PBS, cells were fixed with acid ethanol (95% ethanol: 5% acetic acid) for 10 minutes at room temperature. Samples were then washed with distilled water three times and mounted. Apoptotic nuclei were counted at 400x magnification. Within several hours of dying by apoptosis, cells underwent

secondary necrosis (since they were not phagocytosed in these cultures) and detached from the substrate (Bonfoco et al., Proc. Natl. Acad. Sci. USA 92:7162-7166 (1995)). Hence, several hours after apoptotic cell death, dead cells were no longer present to be stained by Hoechst dye. This temporal separation made it possible to distinguish the number of cells recently undergoing apoptosis by using sequential Hoechst staining at different time points. Additionally, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays were performed in a blinded fashion using an In Situ Cell Death Detection Kit tagged with tetramethyl-rhodamine (Roche, Nutley, New Jersey) or an Apoptosis Detection System Kit tagged with fluorescein (Promega Corporation, Madison, Wisconsin).

**EXAMPLE VII**

**INHIBITION OF MEF2 FUNCTION DOES NOT AFFECT CELL DIVISION OF MULTIPOTENT PRECURSOR CELLS**

This example demonstrates that MEF2 transcriptional activity has no significant effect on cell division of multipotent precursor cells.

Multipotent precursor cells are known to proliferate, leading to an expansion of the cell population that can eventually differentiate into neurons. Cells transfected with dominant negative MEF2 were analyzed for an effect on the proliferation of multipotent precursor cells. Control and MEF2 dominant negative cultures were treated with retinoic acid for 3.0 days, followed by addition of BrdU for detection of dividing cells. BrdU-positive cells and multipotent

precursor cells were identified by double labeling with anti-BrdU and anti-nestin antibodies, respectively (Figure 9*E*). The percentage of cells positive for both BrdU and nestin (proliferating, multipotent precursor cells) was similar in the control and MEF2 dominant negative cultures (Figure 9*F*). These results indicate that MEF2 transcriptional activity has no significant effect on cell division of multipotent precursor cells.

Cell proliferation assays were performed as follows. To label proliferating cells, bromodeoxyuridine (BrdU; Amersham Life Science) was added to cultures at a dilution of 1:1000 for two hours at 37°C. After washing with PBS, cells were treated with acid ethanol (95% ethanol: 5% acetic acid) for 30 minutes at room temperature and cellular DNA was denatured with 2N HCl. After an additional wash in PBS, cells were incubated at 4°C overnight in rat monoclonal anti-BrdU antibody (1:10; Harlan Sera-Lab Limited, Indianapolis, Indiana) and mouse monoclonal anti-nestin antibody (11 µg/ml). After three washes in PBS, secondary antibodies were added: Rhodamine Red-X-conjugated anti-rat IgG (1:200) and Biotin-SP-conjugated anti-mouse IgG (1:50; Jackson ImmunoResearch Laboratories, Inc.; Westgrove, PA) followed by streptavidin-fluorescein (1:25; Amersham Life Science Inc.). Cells were examined under epifluorescence microscopy.

## EXAMPLE VIII

## INHIBITION OF P38 MAP KINASE INCREASES APOPTOSIS DURING NEURONAL DIFFERENTIATION

This example demonstrates that the p38α/MEF2 cascade plays a role in preventing apoptotic cell death during neuronal differentiation.

Interference with MEF2 function enhanced apoptosis in differentiating P19 cells, indicating that MEF2 transcriptional activity was necessary for prevention of cell death during neuronal development. Two members of the p38 MAP kinase family, p38α and p38β, are known to activate MEF2 via phosphorylation of Ser/Thr residues (Han et al., Nature 386:296-299 (1997); Zhao et al., Mol. Cell. Biol. 19:21-30 (1999); and Yang et al., Mol. Cell. Biol. 19:4028-4038 (1999)). Moreover, the p38 MAP kinase family can play a role in cell survival in several cell types (New and Han, Trends Cardiovasc. Med. 8:220-229 (1998)).

The p38/MEF2 pathway was examined for a role in preventing apoptosis in differentiating P19 cells. Activation of p38 family members was examined by immunoblotting with an anti-phospho-pan p38 antibody, which recognizes all activated/phosphorylated p38 MAP kinases. One band was strongly induced after retinoic acid treatment, and the mobility of this band was the same as p38α (Figure 10*A*). Total (phosphorylated and unphosphorylated) p38α protein appeared to be present at similar levels before and after stimulation with retinoic acid (Figure 10*A, right-hand lanes*). In contrast, p38β was undetectable with two different antibodies, although

these antibodies clearly reacted with recombinant p38β protein under the same conditions in control studies. These results indicate that p38α is activated/ phosphorylated during the induction of neurogenesis.

Transfection of dominant negative p38α, but not dominant negative p38β, enhanced apoptotic cell death in differentiating cells. Furthermore, co-expression of constitutively active MEF2C significantly rescued these differentiating cells from apoptosis (Figure 10*B*). These findings indicate that the p38α/MEF2 cascade plays a role in preventing apoptotic cell death during neuronal differentiation.

Transient transfection during differentiation of P19 cells was performed as follows. P19 cells ($2 \times 10^5$) were seeded onto 6-well tissue culture plates and treated with 300 pM retinoic acid to induce neuronal differentiation. One day later, cells were transfected with 0.83 μg of a p38 dominant negative construct or vector alone [pcDNA3-p38α(AF), pcDNA3-p38β(AF) or pcDNA3], 0.83 μg of a constitutively active MEF2C construct or vector alone (pcDNAI-MEF2C 1-117/VP16 or pcDNAI-Amp), plus 0.33 μg of a Green Fluorescent Protein (GFP) construct to identify transfected cells (pEGFP-N1). A lipid based transfection system was utilized (6 ml of TransFast, Promega). The next day cells were transferred to 3.5-cm bacterial dishes and treated with an additional 300 pM retinoic acid. TUNEL assays were performed on day three after initiating retinoic acid differentiation. Over 1200 GFP-positive cells were scored for apoptosis from each culture plate under epifluorescence microscopy,

and each experiment was replicated on three separate days.

## EXAMPLE IX

## EXPRESSION OF CONSTITUTIVELY ACTIVE MEF2 IN UNDIFFERENTIATED ES CELLS

*A. Expression of constitutively active MEF2*

Murine embryonic stem cells (ES cell line D3) were plated at a 1:5 dilution in ES Cell Passage Medium (DMEM with 20% fetal bovine serum, 2250 mg/L glucose, MEM non-essential amino acids, 1 mM sodium pyruvate and penicillin-streptomycin) in gelatin-coated 24 well plates. One day later, ES cells were incubated for five hours with a mixture of 1.25 µg of vector alone (pCDNA1) or pCDNA expression vector encoding constitutively active MEF2 (pCDNA MEF2C/VP16), 0.25 µg expression vector encoding green fluorescence protein (GFP; pEGFPN1; Clontech), and 3 µl of LipofectAMINE 2000 (Promega). Transfected cells were identified by expression of GFP. Cells were washed twice with Neuron Induction Medium (DMEM with 10% bovine calf serum, 4500 mg/L glucose, 10% F-12 growth supplement, 1 mM glutamine, 25 mM HEPES (pH 7.0) and penicillin-streptomycin). Further incubation for one day in Neuron Induction Medium resulted in differentiation of ES cells to neurons as indicated by the presence of neuron-specific markers (see below).

*B. Characterization of differentiated ES cells*

Cells were analyzed for expression of the neuron-specific marker, neurofilament H essentially as follows. Cells were trypsinized and transferred into a four-well chamber slide (Nunc) coated with laminin and poly-L-lysine. Two days later, cells were fixed with 3% paraformaldehyde and permeabilized with 0.3% Triton-X 100. Expression of neurofilament H was determined with monoclonal antibody to anti-neurofilament H monoclonal antibody SMI311 (Sternberger Monoclonals) and detected with anti-mouse IgG conjugated to rhodamine Red-X (Jackson ImmunoResearch). Red (neurofilament H-positive, transfected (GFP-positive) cells were scored using epifluorescence microscopy. The results showed that eighty-three percent of transfected cells displayed expression of neurofilament H, indicating a high degree of efficiency in conversion of murine ES cells transfected with constitutively active MEF2C to neurons. Similar results were obtained with ES cells transfected with constitutively active MEF2A/VP16.

These results demonstrate that overexpression of constitutively active MEF2 (MEF2/VP16) induces neurogenesis in undifferentiated ES cells.

All journal article, reference, and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference.

Although the invention has been described with reference to the examples above, it should be understood

that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.